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Treatment aspects, prognostic factors and outcome measures of lymphatic malformations

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Nicole and Estelle,

A person without a dream is a person,

who will not be able to grow out wings.

That person will never be able to fly.

I want to see you fly.

Fly and don't let them ever wake you up from your dreams.

There will be a lot of times that they will want to wake you up,

Listen to your dad, in life only the dreamers are fully awake…

…Baba Nader

ABSTRACT

Lymphatic malformations (LMs) are structural defects in the lymphatic vessels.
LMs are histologically benign lesions, however, due to localization, size and unexpected swelling, they may cause serious complications that threaten vital functions such as compression on the airways. A large swelling of the face or neck may also affect the esthetics and thus constitute a psychological strain for the patients and their families. LMs are also highly immunologically reactive and are prone to recurrent infections and inflammation causing pain as well as chronic oozing wounds.
There are no available guidelines that describe management and follow up of LMs. Surgery has previously been considered the first treatment option. Surgery, however, has limitations as LMs often infiltrate adjacent structures, such as vessels and nerves making total resection difficult and potentially dangerous. Percutaneous sclerotherapy has replaced surgery as the primary treatment in most cases. Sclerotherapy also has limitations and can potentially cause serious complications such as unpredictable swelling that affects vital functions and cause scarring in the surrounding tissue.
Review of the literature raises several questions. It is still unclear, which should be the treatment criteria for LMs and how to follow up the patients. LMs are rare conditions, however, our department has accumulated a large cohort of patients. No other institution has published a larger cohort of patients with LMs.

The main objectives of this project was to evaluate the surgical- and interventional outcome of the treatment of LM patients, identify prognostic factors and better understand the inflammatory mechanisms of OK-432 treatment.

In this dissertation the following research questions were addressed.

1. Are there immunological pre-requisites that can be analyzed in a blood sample that provide prognostic information on the outcome of sclerotherapy using OK-432?

2. How good is the long-term clinical outcome and the health-related quality of life of patients treated with sclerotherapy using OK-432?

3. Is it possible to establish a treatment algorithm for patients with LMs involving the mediastinum by evaluating the current management with both surgery and sclerotherapy?

In **Paper I** the hypothesis was that Toll-like receptor (TLR) expression in monocytes after treatment with the TLR4-ligand lipopolysaccharide (LPS) could be used to predict successful OK-432 treatment. Blood was analyzed from children with low response (LR, n = 6) and high response (HR, n = 5) to previous OK-432 sclerotherapy. Monocytes were stimulated with LPS. TLR4 expression was analyzed with fluorescence-activated cell sorting (mean fluorescence intensity (MFI)). The mean TLR4 upregulation after LPS stimulation was 3.6 times higher in the HR group than in the LR group and non-stimulated controls (P = 0.037). Dynamic TLR4 expression most probably represents a predictive parameter for the treatment of LMs with OK-432. In **Paper II,** further analysis showed that the mean expression of TLR 4 after LPS stimulation was comparable in both groups (HR 1142 ± 652 units, LR 839 ± 427 units, P = 0.85). The pre-stimulation values in the LR group compared with the HR group were 950 ± 718 vs. 477 ± 341 with considerable differences of the mean expression changes after LPS stimulation (HR 665±683vs.LR 111±605,P=0.08). The difference in TLR4 upregulation on monocytes after LPS stimulation in the LR group compared with the HR group can be explained by TLR pre-conditioning. The findings suggest that absolute threshold values of TLR 4 could be a predictive parameter for the treatment of LMs with OK-432.

In **Paper III** demographic data and long-term outcome in patients with LMs treated with OK-432 were analyzed. We enrolled 131 of 138 eligible patients treated with OK-432 for LMs between 1998 and 2013 in a retrospective study. The outcome was assessed with a clinical examination, evaluated with a clinical assessment score (CAS), and a questionnaire. LMs were localized to the head/neck (60%), the trunk (20%) and the extremities (6%) or involved more than one region (14%). Patients with microcystic (10%), macrocystic (21%) and mixed lymphatic malformations (69%) underwent a median number of three, two and two injection treatments, respectively. OK-432 treatment resulted in a successful outcome in 70% of patients with LMs. The long-term outcome was comparable to the short-term outcome. The number of injections, previous treatment and lesion localization predicted the clinical outcome. Four unsatisfactory attempts of sclerotherapy were shown to be a breakpoint for surgery.

In **Paper IV** the management of patients with LMs involving the mediastinum was reviewed and a treatment algorithm was suggested. All patients with LMs involving the

mediastinum between 2009-2015 at our institution were reviewed. We collected demographic data, data on investigations, management, and complications of the treatment, as well as outcomes at follow-up. Complications were described according to the Clavien-Dindo classification. The patients treated with sclerotherapy and the operated patients had comparable numbers of Clavien-Dindo grade I-II complications. Clavien-Dindo grade III-IV complications were five times more frequent after sclerotherapy than after surgery. The clinical outcome was excellent for the operated patients and fair to good for the patients receiving only sclerotherapy. Patients with cervical LM involving the mediastinum represent a high-risk group with respect to the severity of complications following sclerotherapy. Surgical resection of the LM in the mediastinum is recommended, with the possibility of intra-operative sclerotherapy as an adjunctive.

In **Paper V** the health-related quality of life (HRQOL) was assessed in the cohort of Swedish children and adolescents with LMs who underwent injection treatment with OK-432 at our institution between 1998 and 2013. A study-specific questionnaire was sent to all patients with at least five years' follow up after the first injection treatment asking for persisting symptoms and satisfaction with the treatment and care. KIDSCREEN-52 was used to assess HRQOL. Patients with LMs localized in the head and neck area and repeated sclerotherapy constitute a risk for negatively affected HRQOL.

LIST OF SCIENTIFIC PAPERS

I. Reismann M, **Ghaffarpour N**, Luvall E, Jirmo AC, Winqvist O, Radtke J, Wester T, Claesson G. Dynamic Toll-like receptor expression predicts outcome of sclerotherapy for lymphatic malformations with OK-432 in children.
J Surg Res. 2014 Mar;187(1):197-201.

II. Reismann M, **Ghaffarpour N**, Luvall E, Jirmo A, Radtke J, Claesson G, Wester T. TLR4 preconditioning is associated with low success of OK-432 treatment for lymphatic malformations in children.
Pediatr Surg Int, 2016 May:32(5):435-8

III. **Ghaffarpour N**, Petrini B, Svensson LA, Boman K, Wester T, Claesson G. Patients with lymphaticmalformations who receive the immunostimulant OK-432 experience excellent long-term outcomes.
Acta Paediatrica 9 June 2015,104:1169–1173

IV. **Ghaffarpour N**, Mesas Burgos C, Wester T.
Surgical excision is the treatment of choice for cervical lymphatic malformations with mediastinal expansion.
J Pediatr Surg (2017) Avaliable online

V. **Ghaffarpour N**, Claesson G, Wester T, Boman K.
Long-term Health Related Quality of Life in a Cohort of Swedish Children with Lymphatic Malformations Treated with Sclerotherapy.
JAMA Pediatrics (2018) Submitted

CONTENTS

LIST OF ABBREVIATIONS

ADL	Activity of Daily Living
Ang1	Angiopoietin-1
AVM	Arterio-Venous Malformation
CAS	Clinical Assessment Score
CM	Capillary Malformation
CD14	Marker molecule on monocytes
CI	Confidence Interval
DIC	Disseminated Intravascular Coagulation
D2-40	Podoplanin
E12.5	Embryonic day 12.5
EXIT	EX-Utero-Intrapartem Treatment- procedure
FACS	Fluorescence-activated cell sorting
HLA-DR	Human Leucocyte Antigen DR
HR	High Responder
HRQOL	Health-Related Quality Of Life
ICU	Intensive Care Unit
IFN-γ	Interferon gamma pro-inflammatory cytokines
IL-10	Interleukin-10 pro-inflammatory cytokines
IL-17A	Interleukin-17A pro-inflammatory cytokines
ISSVA	International Society for the Study of Vascular Anomalies
KIDSCREEN-52	HRQOL screening instrument
LEC	Lymphatic Endothelial Cells
LIC	Localized Intravascular Coagulopathy

LM	Lymphatic Malformation
TLR	Toll Like Receptor
TLR4	Toll Like Receptor 4
TLR7	Toll Like Receptor 7
LPS	Lipo-Poly-Saccharide
LR	Low Responders
LYVE	LYmphatic VEssels Hyaluronan receptor
Macro LM	Macro cystic Lymphatic Malformation
Micro LM	Micro cystic Lymphatic Malformation
Mixed LM	Mixed Lymphatic Malformation
MFI	Mean Fluorescence Intensity
MRI	Magnetic Resonance Imaging
mTOR	Mechanistic target of rapamycin
NSVA	Nordic Society for the study of Vascular Anomalies
NICU	Neonatal Intensive Care Unit
OK-432	Picibanil
p-value	Probability value
PDGF	Platelet-Derived Growth Factor
PDGF-R	Platelet-Derived Growth Factor receptor
PICU	Pediatric Intensive Care Unit
PIK3CA	Gene coding for the enzyme PI3K
Prox1	Homebox gene
RASA1	Gene coding for Protein activation
SD	Standard Deviation

SEM	Standard Error of the Mean
S1P	Sphingosine-1-Phosphate
Tie 1 and Tie 2	Angiopoetin receptors
TGFb	Transforming Growth Factor beta
TGFbR	Transforming Growth Factor beta Receptor
VascERN	Vascular European Reference Network
VEGF	Vascular Endothelial Growth Factor
VEGF-R	Vascular Endothelial Growth Factor Receptor
VM	Venous Malformation
WHO	World Health Organization

1 PROLOGUE

The field of vascular anomalies and lymphatic malformations needs to be further explored. Vascular anomalies represent a spectrum of disorders from a simple “birthmark” to life-threatening entities. Patients with these anomalies commonly experience misdiagnoses due to confusion in the nomenclature. Accurate diagnosis is crucial for appropriate evaluation and management, which often requires a multidisciplinary approach.

At the Department of Pediatric Surgery at Karolinska University Hospital, we have been faced with this demanding group of patients for many years. Our largest experience over the years concerning patients with vascular anomalies has been patients with lymphatic malformations (LMs). This doctoral thesis is focused on LMs. The objective was to better understand these complex malformations and evaluate the management.

Prior to launching of this project we had some general philosophical and ethical considerations. First of all, the number of subjects is limited due to the fact that this is a rare condition. The philosophical question whether it is ethically correct to study such a small field, when the possible benefit from the study will affect such a limited number of individuals, may be mandated. This is a matter of utilitaristic considerations whether the benefit of all science should aim at the good of all mankind or if the good of the individual is good enough. From one aspect we all share the same resources and we should aim to use our resources for the best of as many as possible. The other aspect is that we have a responsibility to try to handle the suffering of minorities and the ones with weak voices. Second, we have to bear in mind that we may find correlations between individual risk factors, potential co-morbidity, and mortality. This fact is also a matter of philosophical and ethical reflections. It is a balance between the good in finding a reason to intervene and protect an individual against a hazardous event in the future and the principle of autonomy where a person may not want to know about future events. A third general matter to consider is that the cohort consists mostly of children. Children are a vulnerable group of patients with somewhat limited possibilities to take part in a study and give an autonomous approval to a study in a written informed consent. Small children up to a certain age will have their legal guardians who will give their approval for the child to participate in a study. Mature children and grown-ups will give approval by themselves. The group of patients in between are the most problematic, when asked to participate in scientific studies. The legal age in Sweden when a child is heard is twelve, however, children much younger

than twelve have the right to protect their individual principle of autonomy themselves. The possibility of a child participating in a study due to the will of the doctor or the parents must be taken into consideration and the child's rights should be protected.
The Regional Ethics Review Board in Stockholm approved the studies in this thesis. To the best of my knowledge we have conducted our studies based on solid ethical and scientific foundations.

1.1 HISTORICAL REFLECTIONS

Throughout the history and in all cultures birthmarks have had mythological and superstitious explanations. The take on these congenital spots vary from good and luck bringing to being a very bad stigma for the affected individual. Many societies once believed that a pregnant woman's behavior could cause birthmarks. Some believe that eating too many red foods caused strawberry marks. Others believe that strong fearful emotions or overwhelming desires of a mother imprinted the marks. In some cases, people believe that the marks indicate an area where a person was injured in a previous life. In Iranian lore, it is thought that if a woman touches her belly as she watches a solar eclipse, her baby will be born with a birthmark. Some interpretations of birthmarks are more insidious. People with birthmarks have been cast out of normal society and demonized as witches because they were born with the "devil's mark." Fortunately, not all birthmark legends have such negative connotations; in some eastern European countries, it's considered good luck to touch someone with a birthmark. But even without superstitions and myths adding to the confusion, birthmarks often come with heavy emotional baggage. Many people ridicule children with disfiguring birthmarks, leaving psychological wounds much deeper than the marks themselves.

All since the 17-th century individuals with congenital disfigurements have been humiliated in organized freak shows to amuse the crowds. One example, commonly known, is Joseph Merrick, "The Elephant man" (Fig 1), who probably had Proteus syndrome with disfigurement caused by vascular anomalies. Another example may be Quasimodo, the character from the Novel "The Hunchback from Notre Dame" written by the novelist Victor Hugo, who describes an individual who probably had LMs on his face and back. These individuals have been ridiculed and humiliated in circus shows due to their congenital malformations and in between the exposures they have been hidden from society and abandoned by their families [1].

Fig 1 Fannie Mills ” The Ohio Big Foot Girl”, and ” John Merrick ”The elephant man” two persons who have suffered the humiliation of being objects in side-shows due to congenital malformations[2].

2 INTRODUCTION

LMs are rare anomalies of the lymphatic vessels. In order to improve and develop the management of LMs it is essential to understand the mechanisms behind the pathophysiology and the clinical problems as well as the normal physiology and embryology. LMs are defined as structural defects due to abnormal embryonic or fetal development. The clinical problems are heterogeneous.

Vascular embryology

The initial stages of vascular development are best described by the terms vasculogenesis and angiogenesis. The two processes describe the formation of new vessels by mesodermal precursor cells (Fig 2)[3, 4]. Angioblasts migrate, to specific sites in the embryo, adhere to one another and form loose connections. Later, the vascular tree grows by sprouting, cell division, migration, and assembly of endothelial cells derived from preexisting vessels through a process termed angiogenesis[3].

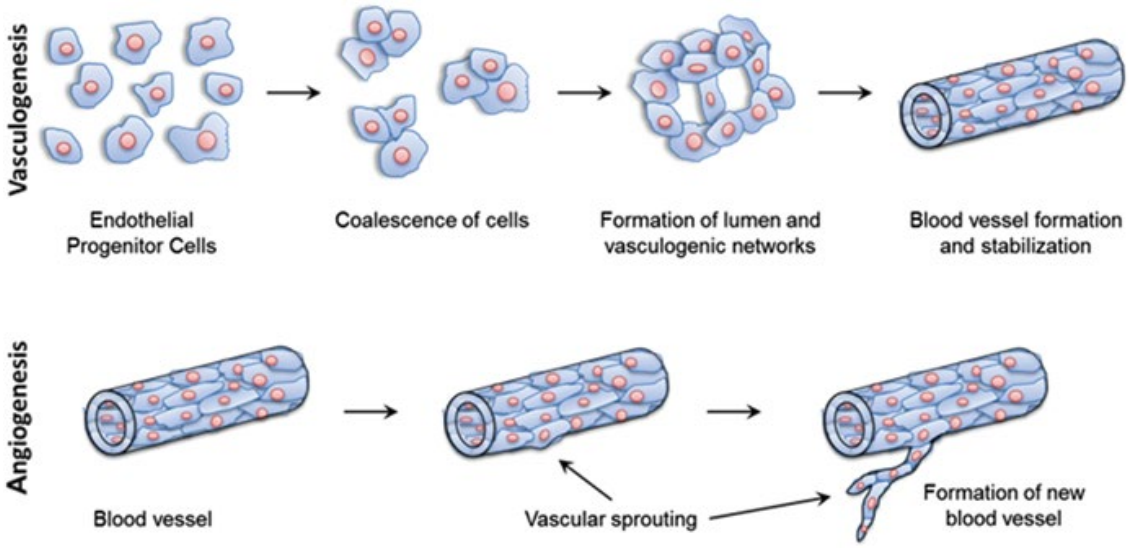


Fig 2 New blood vessel formation occurs via vasculogenesis and angiogenesis[5].

The molecular mechanisms that control the development of the vascular system are partly unraveled[6, 7].

The vascular endothelial growth factor (*VEGF*) and the corresponding receptor VEGF-R seem to play a crucial role in the early phase of the vasculogenesis, whereas another family of vascular endothelial growth factors (Angiopoietins) and their receptors, *Tie1* and *Tie2*, appear to play a latter role by controlling the sprouting, remodeling, and maturation of the developing vasculature (Gale and Yancopoulos, 1999)[7] (Fig3).

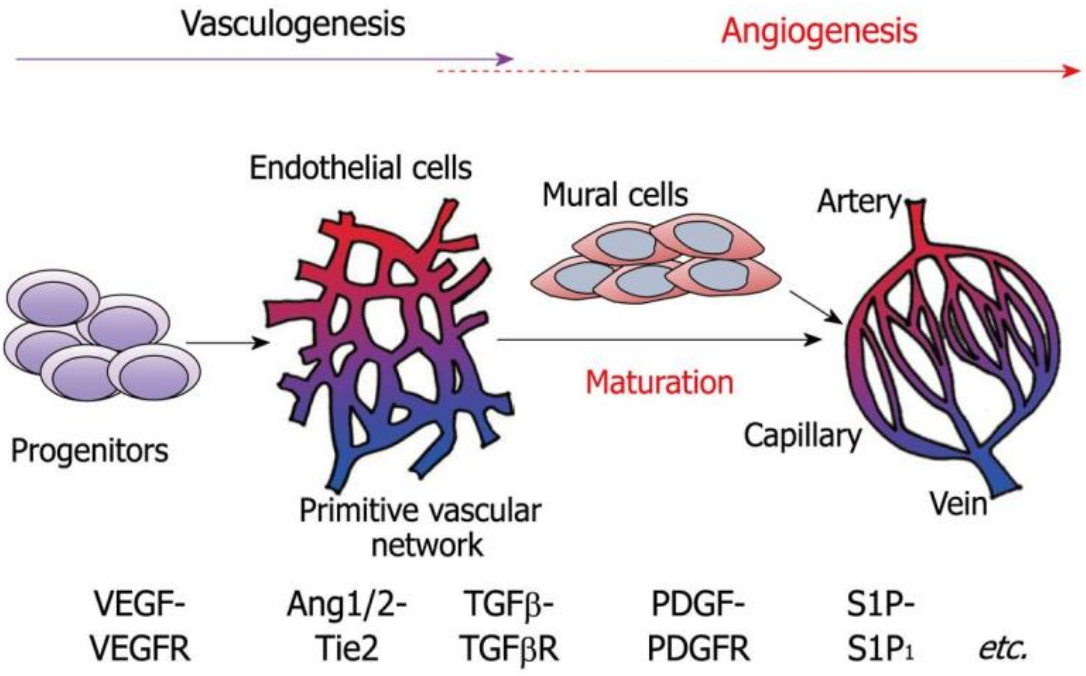


Fig 3 Schematic representation of vascular formation and the role of the growth factors during vasculogenesis and angiogenesis[5].

Lymphangiogenesis

Lymphangiogenesis is the development of lymphatic vessels (Fig 4). The mechanisms controlling the development of the lymphatic vascular system are largely unknown. The lymphatic vessels form, like the blood vessels, from hemangioblastic stem cells. The first signs of developing lymph nodes are found already in the 5th gestational week[8, 9].

The lymphatic system is a vascular network of thin walled capillaries and larger vessels lined by a continuous layer of endothelial cells that drain lymph from the tissue spaces of most organs and return it to the venous system for recirculation. The origin of the lymphatic vessels is still controversial. Historically, the most widely accepted view of lymphatic development is the one originally proposed by Sabin (1902, 1904)[10].

Sabin proposed that early in development isolated primitive lymph sacs originate from endothelial budding from the veins. Sabin's model proposed that from these primary lymph sacs the peripheral lymphatic system then spreads by endothelial sprouting into the surrounding tissues and organs. (Sabin, 1902, 1904; Gray, 1985)[10]. A report of the vascular endothelial growth factor receptor-3 expression pattern (*VEGFR-3*) during murine development [11] has provided support for Sabin's model of lymphatic development. At embryonic day 12.5 (E12.5), *VEGFR-3* is expressed in developing venous and early lymphatic endothelia, and it appears to become largely restricted to lymphatic endothelium in the adult tissues[11].

These findings suggest that *VEGFR-3* may play a role in the development of the lymphatic system. It has been demonstrated that the homeobox gene *Prox1* is a specific marker of a subpopulation of endothelial cells that by budding and sprouting gives rise to the murine lymphatic system. These findings fully validate Sabin's proposal of the venous origin of the primary lymph sacs (Sabin 1902, 1904). Lymphatic vessels are thought to originate from the venous system, and this process is critically regulated by *Prox1* transcription factor[12].

This mutual embryological background of the venous and the lymphatic systems provide theoretical explanations to the clinical observations of malformations with venous as well as lymphatic components mixed in the malformed tissue.

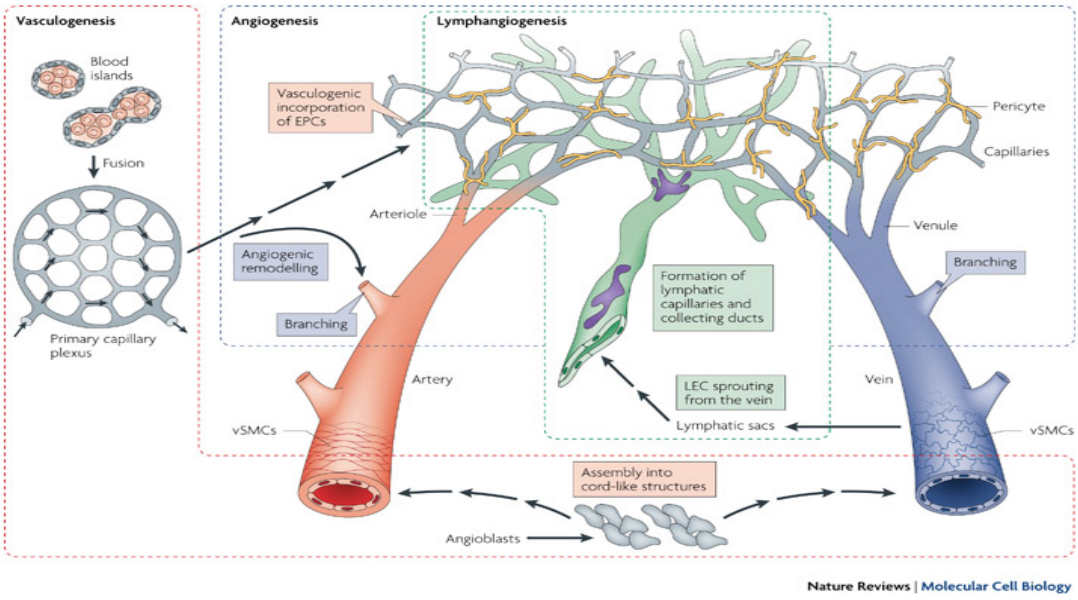
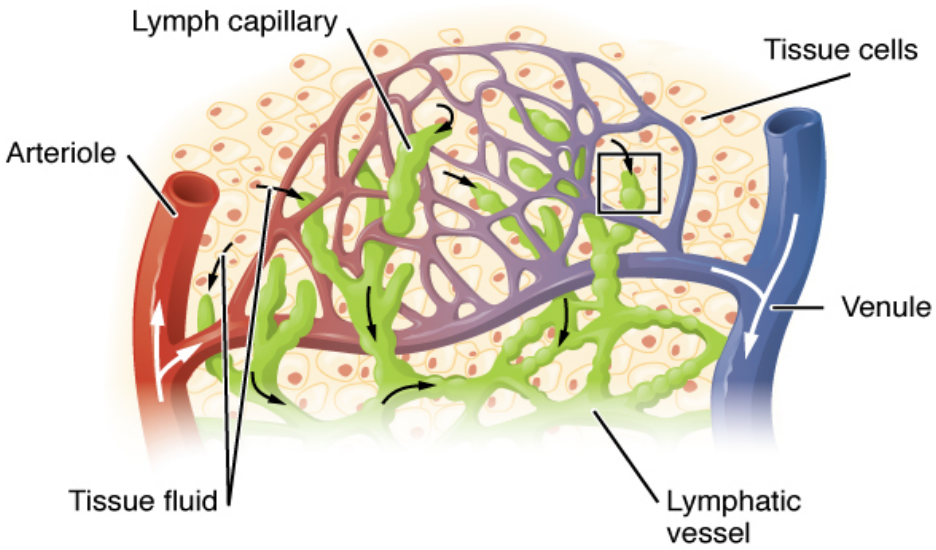


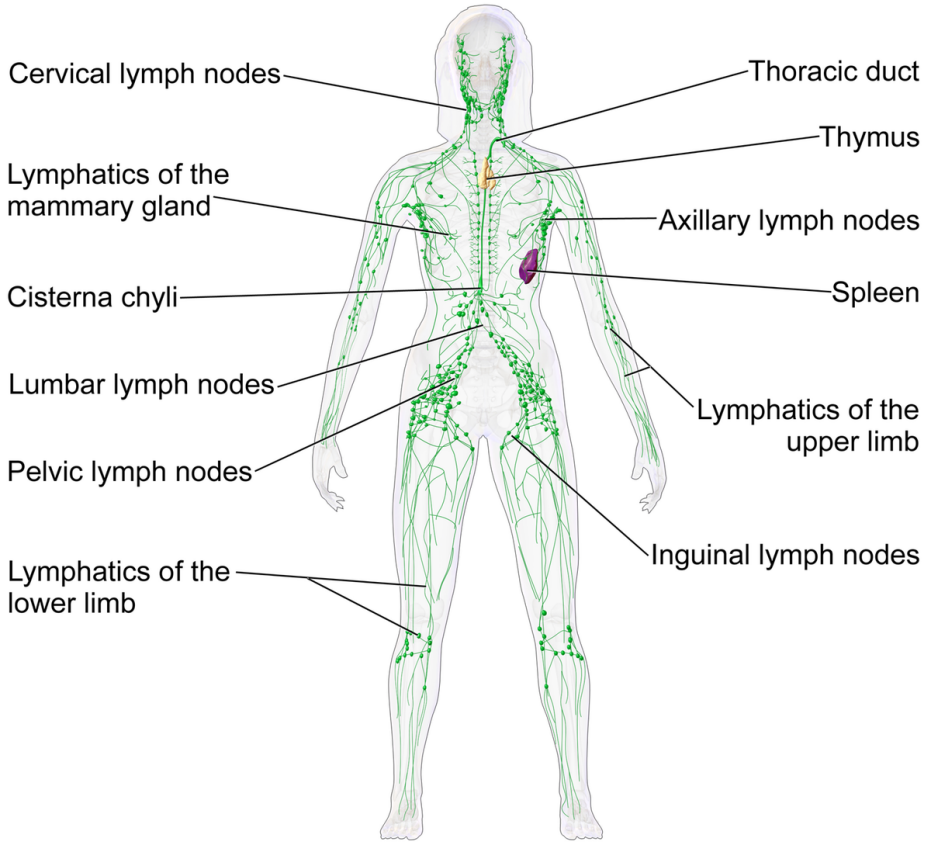
Fig 4 The first lymphatic endothelial cells (LECs) sprout from the embryonic veins, then migrate and form lymphatic sacs. Further steps of lymphangiogenic growth involve sprouting, branching, proliferation, differentiation and remodeling processes[13].

The role of the lymphatic system

The Swedish scientist, writer and professor of medicine at Uppsala University Olaus Rudbeck first described the lymphatic system in the seventeenth century. He described a watery fluid distributed in fine vessels throughout the body parallel to the blood vessels. The lymphatic system consists of lymphatic organs, a conducting network of lymphatic vessels, and the circulating lymph (Fig 5). The lymphatic vessels include the tubular vessels of the lymph capillaries, and the larger collecting vessels the right lymphatic duct and the thoracic duct. The lymph capillaries are mainly responsible for the absorption of interstitial fluid from the tissues, (Fig 5) while lymph vessels propel the absorbed fluid with smooth muscle contractions forward into the larger collecting ducts, where it ultimately returns to the bloodstream via one of the subclavian veins. Through the network of capillaries and collecting lymphatic vessels from all parts of the body interstitial fluid is efficiently drained and extravasated fluid is transported, along with large proteins and antigens, back to the circulatory system. Numerous intraluminal valves in the vessels ensure a unidirectional flow of lymph without reflux.



a



b

Fig 5 Schematic overview of the **a** interstitial (tissue) fluid (the lymph) surrounded by lymph capillaries and the[13] **b** lymphatic system including the lymphatic organs, the lymphatic vessels and the lymph nodes[14].

The main function of the lymphatic system is:

1. To remove interstitial fluid including cellular waste products and cellular debris together with invading bacteria and large proteins from the peripheral tissues.
2. To absorb and transport fatty acids and fat as chyle from the digestive system
3. To transport antigen-presenting cells such as dendritic cells to the lymph nodes along with bacterial and viral debris where an immune response is stimulated and cytokines and interleukins are produced and transported to the circulation.

Formation of vascular malformations

The formation of vascular malformations is highly multi-factorial. However, VEGF is important for the vascular growth phase. It plays an essential role during the angiogenesis, initiating the sprouting and formation of new vessels. Folkman and Haudenschild first observed angiogenesis in vitro in 1980[15, 16].

Angiogenesis does not involve differentiation of the various endothelial cells, but rather the reorganization of an existing vascular network in response to some locally acting angiogenic factors. Whereas VEGF is important for the growth phase, transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), angiopoietin-1 (Ang-1), and their respective receptors are essential for the stabilization phase. Errors in the early differentiation results in vessels devoid of pericytes which display several abnormalities, including endothelial hyper cellularity, vascular tortuosity and the formation of focal micro aneurysms, altered organization of endothelial cell junctions, increased vesicular transport, and leakage of plasma and blood cells[17].

Classification of vascular anomalies

One of the major challenges in the field of vascular anomalies is the nomenclature. Vascular anomalies involve virtually all fields of medicine since they can occur in all parts of the body and in all organ systems. Considering the clinical complexity of these lesions it is important that all specialists use the same classification. The World Health Organization (WHO) classifications are generally considered as the reference classification for tumors and tumor-like diseases. The WHO classification of skin vascular “tumors” is a nonhierarchical list of a series of different diseases, irrespective if the nature is tumor due to cellular proliferation,

malformation, reactive, or infectious. [18]

The WHO classifications of soft tissue tumors is thus misleading and confusing[19].

The lack of consistency in the nomenclature makes it difficult for clinicians across specialties to communicate using a common language specific for each entity, and confusion in the naming of lesions creates inaccuracies in scientific advances. It also makes coding and statistical data about the prevalence and incidence of these lesions inaccurate.

For centuries, vascular birthmarks were referred to by common names such as port-wine stains and strawberry marks. Several classifications of vascular anomalies are available; some are general classifications, and others deal with specific organs or tissues or only with vascular tumors or vascular malformations. In 1863, the father of modern histopathology R. Virchow described the histology of vascular nevi[20].

The terms angioma simplex, angioma cavernosum and angioma racemosum were introduced and described the different forms of blood-filled vascular lesions depending on the size and form of the collecting vessels or channels. G. Wegener, a student of Virchow, refined the nomenclature. Wegener studied the lesions and described that some lesions were blood-filled and some were filled with lymph fluid[20].

At this point the classification described vascular lesions depending on fluid content in heme-angioma and lymph-angioma. The subgroups simplex, cavernosum, and racemosum were suggested to describe the form of the collecting channels. This classification system was widely accepted. Still this nomenclature is used in many institutions and histo-pathology departments. The works done by Virchow and Wegener suggested that all these lesions were tumors, benign of origin, but nevertheless tumors, hence the suffix ¨–oma¨. The modern era of vascular anomalies started in 1982 when Mulliken and Glowacki showed that vascular lesions could clinically look similar, although the endothelial characteristics differed[21].

The classification now divided vascular anomalies in two major fields, *vascular tumors* and *vascular malformations*. The tumors are structures derived from endothelial cell proliferation with an accelerated mitotic activity. They may be benign, intermediate, or malignant depending on the cellular behavior. Vascular malformations occur due to errors in morphogenesis of the vessel formation and exhibit normal mitotic activity of the endothelial cells. The morphogenesis of capillaries, arteries, veins, or lymphatics is deranged and the pooled fluid, whether blood or lymph fluid, is a result of collapsed, dysfunctional, malformed fluid conducting vessel structures. Depending on the dominating type of vessels within the

malformation the flow characteristics of the lesion differ from low-flow to high-flow. Low-flow malformations are capillary malformations, venous malformations and lymphatic malformations and high-flow malformations are arterio-venous malformations and arterio-venous fistulas. The lump in a malformation has no solid component.

Another classification, the Hamburg classification, used vessel type as the basis of classification of vascular malformations. Each vessel type may present malformations within large vessels “truncular malformations” or “extratruncular malformations” composed by smaller vessels diffusely infiltrating and being intimately embedded in the host tissue. This distinction is clinically relevant, because “truncular” malformations seems to behave differently, and are more often associated with pulmonary embolism when affecting veins and with chylous effusion when affecting lymphatic vessels[22].

The International Society for the Study of Vascular Anomalies (ISSVA) was founded in 1992 and one of the main perspectives of the society is to achieve a uniform classification for vascular lesions that can be adopted by all involved specialities.

The current and latest classification was accepted by ISSVA in 2014[23] (Fig 6).

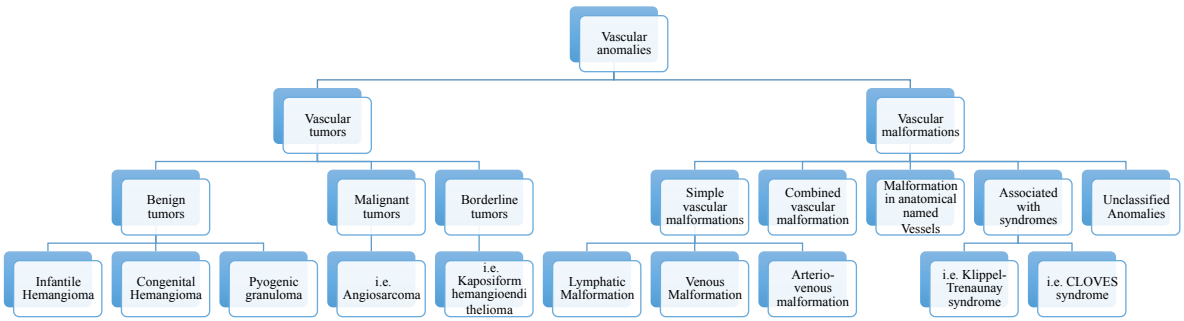


Fig 6 Schematic overview of 2014 ISSVA classification

In the latest ISSVA classification, the Hamburg classification and the original classification of vascular anomalies initiated by Mulliken and co-workers in 1996 have been united. The malformations are classified according to the origin of the vessel, anatomical localization, length, diameter, valves, communication and the persistence of embryonic circulation. The genetic basis of many types of vascular malformations has been further explored and additional disease entities have been identified that need more precise classification rather

than generic headings such as capillary malformation (CM), venous malformation (VM), lymphatic malformation (LM) and arterio-venous malformation (AVM). In the latest classification mixed forms of the malformations has been more accurately recognized and listed[24]. The classification also recognizes a number of dysmorphic syndromes that are associated with vascular anomalies such as Klippel Trenaunay Syndrome, Parkes Weber Syndrome, Servelle Martorell Syndrome, Sturge Weber Syndrome, Maffucci Syndrome, Cloves Syndrome, Proteus Syndrome and Bannayan Riley Ruvalcaba syndrome[24].

Lymphatic malformations

LMs are defined as structural defects that occur because of defective lymphangiogenesis [25]. The incidence of these malformations is 1:6000 to 1:16.000 live births[26]. There are no sexual or ethnic predilections. Ninety-five percent of LMs are diagnosed before 2 years of age. Almost all affected individuals are children. However, occasionally the LM may not be clinically evident until adulthood[25, 27].

The cause of LMs is multifactorial and the exact mechanism is unknown. In many patients genetic analysis of the malformed tissue reveals an activating mutation in the PIK3CA gene. This is a somatic, non-inherited, mutation engaging the lymphatic endothelial cells lining the malformation. PIK3CA is known to play a role in regulating cell growth by signaling through the PI3K/mTOR pathway[28, 29].

It has also been shown that vascular dysmorphogenesis may be caused by an activating mutation in the receptor tyrosine kinase TIE2 and it may also be associated with RASA 1 gene mutation[30, 31].

According to the ISSVA classification LMs are classified as a single vessel malformation, however, combined variants with other vessel structures occur frequently especially in combination with venous malformation since the two vessel types have mutual origin[11, 13].

Histologically, LMs are composed of thin-walled vascular channels lined by a single layer of flattened endothelium. The endothelial cells of the LM are immunopositive for podoplanin (D2-40) and Lymphatic Vessels Hyaluronan receptor (LYVE)-1[32].

The lumen of the lymphatic cysts are filled with immunological active proteins and cells such as interleukines, cytokines, macrophages and lumphocytes[33].

The presence of immunological active proteins makes the lymphatic malformed tissue highly reactive. Blood can also fill the channels indicating spontaneous or traumatic intralesional bleeding or it may indicate mixed form of veno-lymphatic malformation.

2.1 CLINICAL PRESENTATION

The pooled lymph within the malformation expand the affected tissue and causes the clinical symptoms of LMs. LMs may clinically present in a variety of forms, including cystic lymphatic lesions presented as slowly expanding lumps infiltrating the surrounding tissue, angiokeratoma, chylus leak conditions, osseous lesions, generalized systemic lesions part of syndromes or lymphedemas. LMs do not grow by endothelial proliferation, however, they get larger with the same growth pattern as the child. Hormonal changes, trauma, and bleeding may cause them to swell rapidly. Syndromes such as Turner, Noonan, and trisomies are associated with intrauterine LMs.

Usually LMs occur in one isolated area of the body as a subcutaneous lump, occasionally the malformation is more diffusely spread over larger areas of the body, which is then often referred to as lymphangiomatosis. Superficial, palpable LMs expand into deeper cavities in 6% of the cases[34].

All these variants fit into three morphologic subtypes of LMs: Macrocystic, microcystic and mixed lesions (a mixture with both macro- and microcystic components). The specific symptoms and severity of LMs vary based on the size and specific location of the malformation. Macrocystic LMs generally form soft, large, translucent masses. When present in the subcutaneous tissue the overlying skin may have a bluish hue. Macrocystic LMs can potentially be extremely large, even large enough to obstruct the airway at delivery requiring an EXIT (EX-Utero-Intrapartem Treatment- Procedure to secure the airways prior to the delivery.

Microcystic LMs may appear as several small, raised sacs (vesicles) on the skin that contain clear or bloody (hemorrhagic) fluid. They generally grow slowly, usually in proportion with a growing child. Microcystic lLMs can thicken or swell causing enlargement of surrounding soft tissue and bones. They can be found on any area of skin or mucous membrane.

Seventy-five percent of the lesions occur in the head and neck region[35, 36].

Histologically, LMs are benign, however depending on localization, size and potential swelling they may cause serious complications like airway compromise, visual disturbance, pain and motility dysfunction. When evident at birth, LMs tend to be soft, spongy, non-tender masses.

A large swelling in the face or neck may cause esthetic problems and may be a psychological burden to the patients and their families. LMs are highly immune-reactive and are prone to recurrent infections. When LMs become inflamed, they swell, and the skin in the involved area becomes red and warm. Recurrent infections or inflammations cause pain and disfigurement of the affected area. Large infected LMs may also present with septic shock as a massive and sudden auto-infusion of highly immunologically active proteins may occur when pooled infected lymph is shunted into the general circulation from within the infected LM.

Some anatomical regions such as the mediastinum are of additional concern[37]. The mediastinum is a limited cavity with vital structures such as the airways, the large vessels and the heart that may be compressed resulting in a life-threatening complication as the LM expands. LMs affecting the gastrointestinal tract or pelvis can cause constipation, bladder obstruction, recurrent bowel infection or protein loss[38, 39].

LMs may be associated with the hematological disorder, Localized Intravascular Coagulopathy (LIC) with elevated D-dimer and mild to moderate thrombocytopenia. The coagulopathy may progress to Disseminated Intravascular Coagulation (DIC) after trauma or surgery.

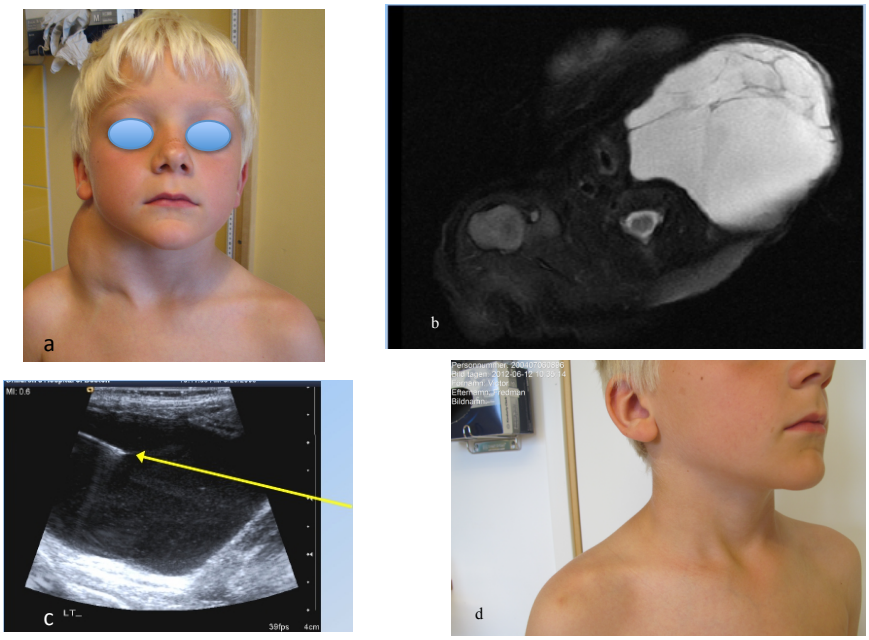


Fig 7 Macrocystic LM **a** clinical features, **b** MRI T2 weighted image of large fluid filled cysts, **c** Ultrasound guided puncture of cysts and injection of OK-432, **d** Clinical outcome after one sclerotherapy session.

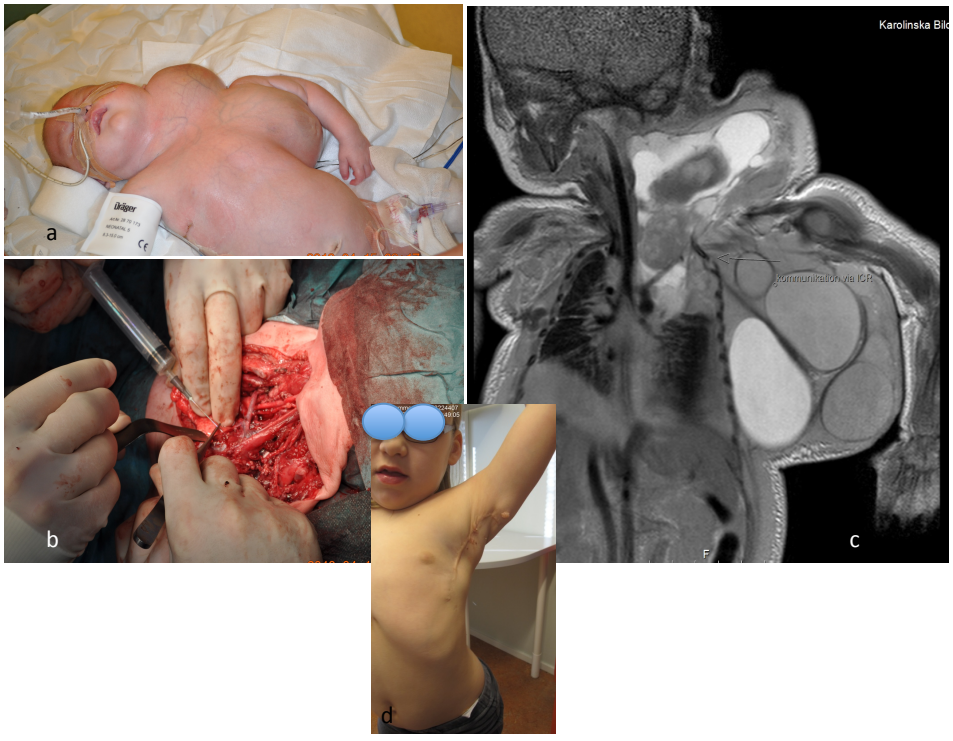


Fig 8 Mixed LM with mediastinal expansion **a** clinical features at EXIT assisted delivery, **b** surgical debulking around the vital structures in the mediastinum and intraoperative sclerotherapy, **c** MRI T2 weighted image showing mixed LM with cysts expanding into the mediastinum compressing the heart and the left main bronchus **d** clinical outcome after stages surgeries and adjuvant sclerotherapies.

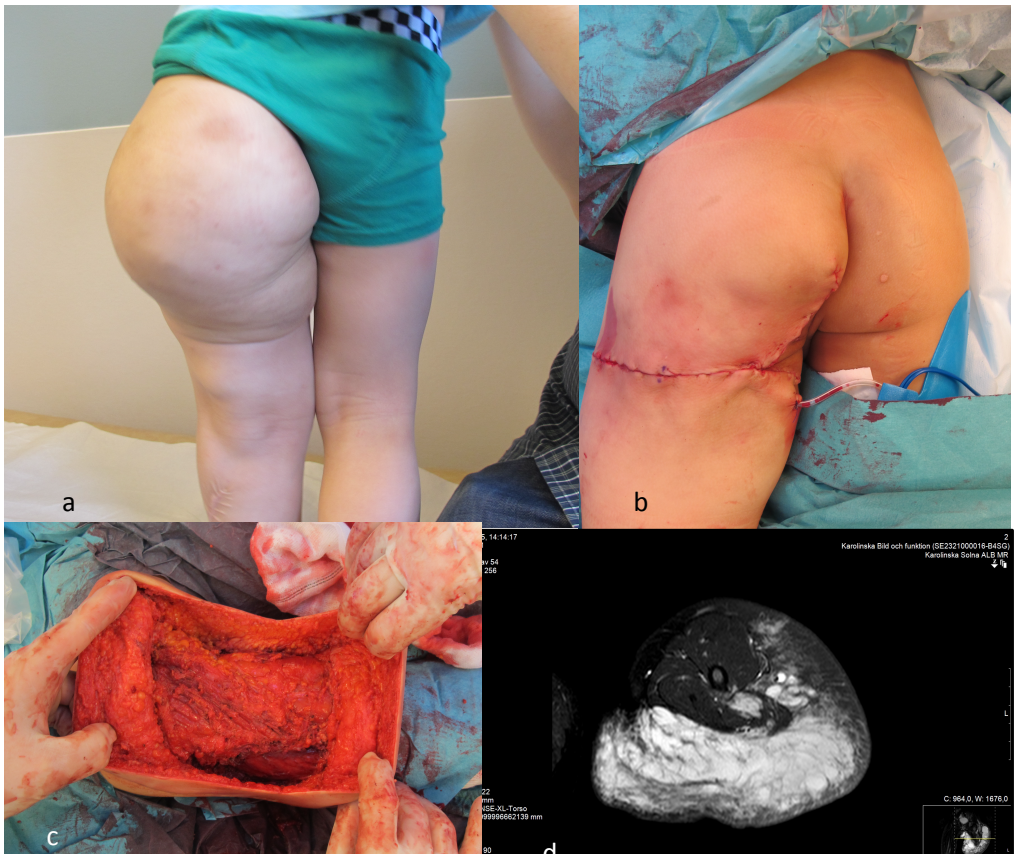


Fig 9 Microcystic LM **a** Clinical features in the gluteal region, **b** After surgical debulking, **c** Intraoperative view after resection of micocystic LM, **d** MRI T2 weighted image showing microcystic LM.

2.2 INVESTIGATION

The diagnosis of LMs can often be made before birth using ultrasound. After birth, a diagnosis of a lymphatic malformation is made based on a physical examination along with a detailed patient history. Ultrasonography is the imaging modality of choice to start the investigation of vascular anomalies (Fig 10). With sonography the flow characteristics of the lesion is measured and the tissue content is evaluated. Many times sonography will give required information about the lesion and be diagnostic. Magnetic Resonance Imaging (MRI) is however the golden standard modality of investigation for vascular anomalies in general to determine the extent and type of LM[40, 41].

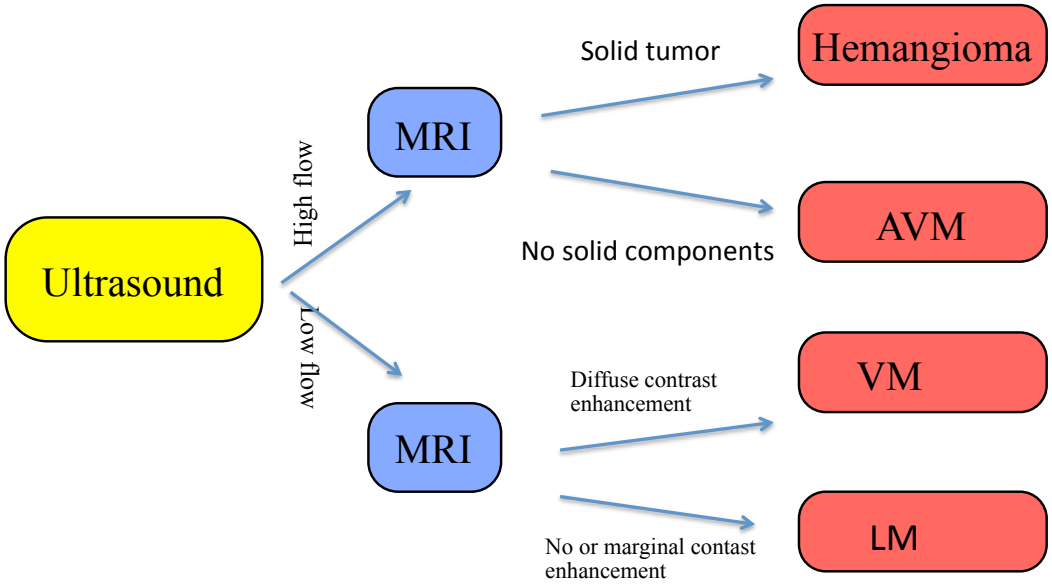


Fig 10 Investigation algorithm for vascular anomalies

In radiology LMs have been categorized into three subtypes – macrocystic, microcystic, or mixed (a combination of the other two) [42](Fig 11). Macrocystic and microcystic LMs are differentiated by the size of the cysts that the malformation consists of. The macrocystic type is made up of a single or multiple fluid-filled cysts, which are all larger than 2 cm in diameter; the microcystic type is made up of smaller cysts or soft tissue enlargement without cyst formation. Most LMs have both macrocystic and microcystic portions[43].

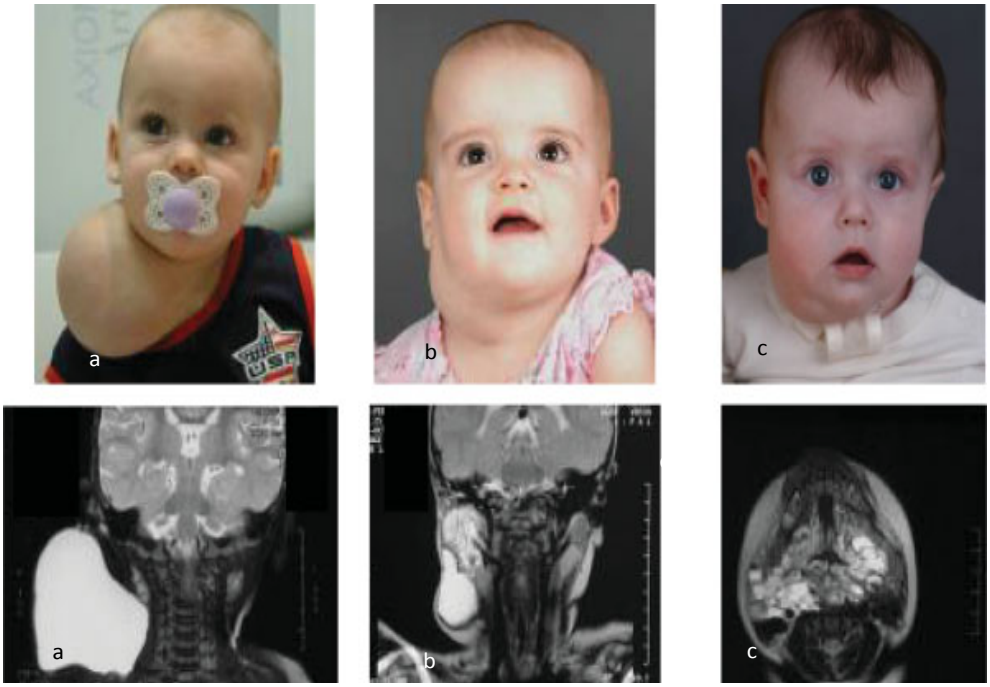


Fig 11 Clinical appearance and corresponding type of LM, **a** Macrocystic, **b** Mixed and **c** Microcystic[42]

Fine needle aspiration cytology is mandated to rule out differential diagnoses such as teratomas, other malignancies, pseudocysts from parynchymal organs, including ranulas from the salivary glands or remnants from brachial clefts.

There are several specific markers available for lymphatic tissue such as D2-40, LYVE-1, PROX-1, desmoplakin and VEGF-C receptor VEGFR-3[32, 44].

These markers can be stained for on tissue samples and examined in clinical practice if diagnostic difficulties occur. This is, however, rarely mandated.

2.3 MANAGEMENT

There is no available treatment algorithm for the management of LMs due the heterogenic presentation among patients. The essential strategy of the treatment of LMs is directed toward the specific symptoms that are apparent in each individual. Symptomatic treatment may be antibiotics as well as painkillers and anti-inflammatory medication. A curative treatment is not always possible and should not be sought necessarily as it may result in too excessive and potentially dangerous treatments. The evaluations of the patients is occasionally complex and require a multi-disciplinary approach involving the insight and

experience of pediatricians, pediatric surgeons, plastic surgeons, ear-nose-throat specialists, radiologists and interventional radiologists among various other health care personnel and paramedics. The specific treatment and interventions vary depending on multiple factors such as type of LM (Macrocystic, microcystic, mixed), size and anatomical localization as well as the presence of certain symptoms such as pain, recurrent infections or oozing. Generally macrocystic LMs can be treated more effectively with better outcome no matter the choice of treatment. Microcystic and mixed LMs are more difficult to treat, often requiring staged and repeated treatments, both interventional as well as surgical. Regardless of treatment modality there is always a risk of recurrence when treating LMs and in some cases LMs treatment is symptomatic and requires life-long therapy.
Rarely LMs may shrink and resolve spontaneously, this is probably induced infection in the malformation that has same effect on the cystic malformations as sclerotherapy.

The main therapeutic options for LMs are watchful waiting, compression garments, percutaneous drainage, surgery, sclerotherapy, laser therapy, radiofrequency ablation, or medical therapy. These different treatment options may be used in various combinations. All treatment modalities aim for the same effect as to remove the spaces were lymph could be pooled in the malformed tissue.

Watchful waiting is an excellent approach after the LM diagnosis is fully established, differential diagnoses are ruled out and the patient and the caretaker have received adequate information. Often watchful waiting is used in small LMs with few or limited symptoms or LMs in situations were the medical problem is not fully evaluated and time will add essential information prior to the decisions for medical or surgical interventions.

Compression garments are the bases of the treatment in lymphedema and microcystic LMs. The malformation, often a limb is wrapped into compression dressings and the pooled lymph is gradually squeezed out from the malformed tissue.

Percutaneous drainage is a limited procedure, which means that the fluid in the LM is drained through a catheter or an incision. Drainage is often used in emergency situations in order to debulk excessive cysts that expand and compress vital structures or functions. The treatment must be combined with sclerotherapy or surgery in order to prevent re-accumulation of lymph in the cyst.

Surgery has traditionally been regarded as the treatment of choice for LM[26, 36].

However, these lesions sometimes present to the surgeon as challenging conditions. Although they are benign in most cases, they frequently, especially microcystic LMs, infiltrate adjacent structures, such as, vessels and nerves[26, 36].

This makes total resection difficult and potentially hazardous. Surgeons are often confronted with serious complications, such as bleeding, wound infection, wound healing problems, nerve damage and recurrence[26]. Large LMs often require staged excisions[35, 45-47]

A multidisciplinary approach involving surgeons and interventional radiologists are often needed for complex LMs. The aim of surgery as treatment for LMs is to remove the lesion and regain function of an affected area and prevent disfiguring complications. Surgery is especially suitable if the LM is localized to one area of the body and if full excision may be performed without sacrifice of vital structures. Surgery has also advantages as part of a staged treatment strategy were sclerotherapy and surgery is combined to maximize debulking of the malformed tissue. With this strategy large areas may be reduced.

Sclerotherapy is a procedure in which an irritant solution is injected directly into the LM. This solution causes scarring within the LM, which eventually leads to shrinking or collapse of the malformation. Percutaneous sclerotherapy has replaced surgery in most cases of macrocystic malformations. During the past 30 years, sclerotherapy has emerged as a promising alternative to surgical management for LMs in children[45, 48-52]. Macrocystic LMs of moderate size can be easily treated with sclerotherapy. Sclerotherapy may require multiple sessions to be effective, especially in extensive malformations.

Many agents have been used for this purpose; among others doxycycline, OK-432, dextrose, bleomycin, ethanol and interferon[52].

A systematic review of the literature on nonsurgical treatment of lymphatic malformations has been carried out. OK-432 and bleomycin sclerotherapy were the most commonly used substances. They showed that 43%, 95%CI (28.9-57%) of patients undergoing OK-432 injections had a complete/excellent response; 23.5%, 95% CI (5.8-41.3%) had a good response; 16.9%, 95% CI (10.3–23.4%) had a fair/poor response; and 15.4%, 95% CI (8.6–22.2%) did not respond. In the bleomycin group the results were as follows: 35.2%, 95% CI (15.7-54.6%) excellent; 37.1%, 95% CI (22-52.3%) good; 18.4%, 95% CI (2.7-34.2%) fair/poor; and 11.6%, 95% CI (3.5-19.6%) no response. The literature strongly suggests that the majority of patients who undergo sclerotherapy with OK-432 or bleomycin as first-line therapy for head and neck LMs will achieve a good to excellent clinical response. Serious complications and the need to progress to surgical salvage were infrequent. Given the heterogeneity of the treatment protocols used and variable results obtained between studies,

there appears to be no clear consensus as to when sclerotherapy is indicated, what agents offer the most benefit, and how these agents should be administered for optimal results[48].

Furthermore, the use of sclerosing agents sometimes causes scarring due to the penetration of adjacent tissues, to the extent that subsequent surgery is difficult or impossible. Disadvantages are the need for repeated injections, skin and soft tissue necrosis, blistering, and to some extent unpredictable swelling with the risk of causing obstruction of vital structures after discharge from the hospital.

Although sclerotherapy for LMs is minimally invasive and often safe, complications may occur ranging from mild systematic symptoms such as fever and fatigue to local swelling causing compression to vital functions requiring prolonged intensive care.

Laser therapy and **radiofrequency ablation** are techniques that energy is deployed in the tissue in order to destroy affected lymphatic vessel tissue and induce shrinkage. These techniques are best suited for superficial skin or mucosal LM[53].

Medical therapy has recently gained additional attention. Sirolimus and Sildenafil can be used to treat both diffuse as well as localized LMs and are administrated orally. Sirolimus has been used in cancer treatment as well as for graft versus host prevention after organ transplantations for a long time. The use for LMs has just recently been recognized and promising reports have been published[54, 55]. Sirolimus acts as a mTOR inhibitor and targets the LM as it inhibits cell growth by targeting the PI3K/mTOR pathway[28, 54, 56]. Additional research is required to fully develop treatment protocols with understanding of treatment duration as well as long-term outcome and side effects. Sildenafil relaxes smooth muscles of the vessel walls, which leads to decreased fluid collection within the LM.

2.4 OK-432

Ogita et al. introduced the immune-stimulant OK-432 as sclerosant for the treatment of LMs in 1987. OK-432 was prepared from strains of *Streptococcus pyogenes* and was primarily certified as an adjuvant cancer therapy in 1975. OK-432 is based on *Streptococcus pyogenes* strains, which are pretreated with penicillin G and heat. This

treatment leads to the loss of the streptolysin S-production. The application leads to various immune-pharmacological reactions that cause tissue shrinkage[57, 58].
The application of OK-432 leads to various immune-pharmacologic events that cause shrinkage of the LM. Experiments with human blood has showed that only one of three individuals is a so called “high responder”, who is able to up-regulate TLR-7 receptor on the leucocytes after OK-432 exposure[59].
OK-432 is acting as a TLR-4 receptor ligand and thus, the activation of TLR-4 will lead to an up-regulation of the TLR-7 receptor, this leads to an expression of sequentially regulatory and pro-inflammatory cytokines like IL-10, IFN-γ and IL-17A[60] (Fig 12).

So called “low responders” produce only IFN-γ, which is just dependent on TLR-4 expression without expression of TLR-7. The latter exhibits a limited immune answer to pro-inflammatory stimulation with TLR-4 ligands[61] (Fig 12).

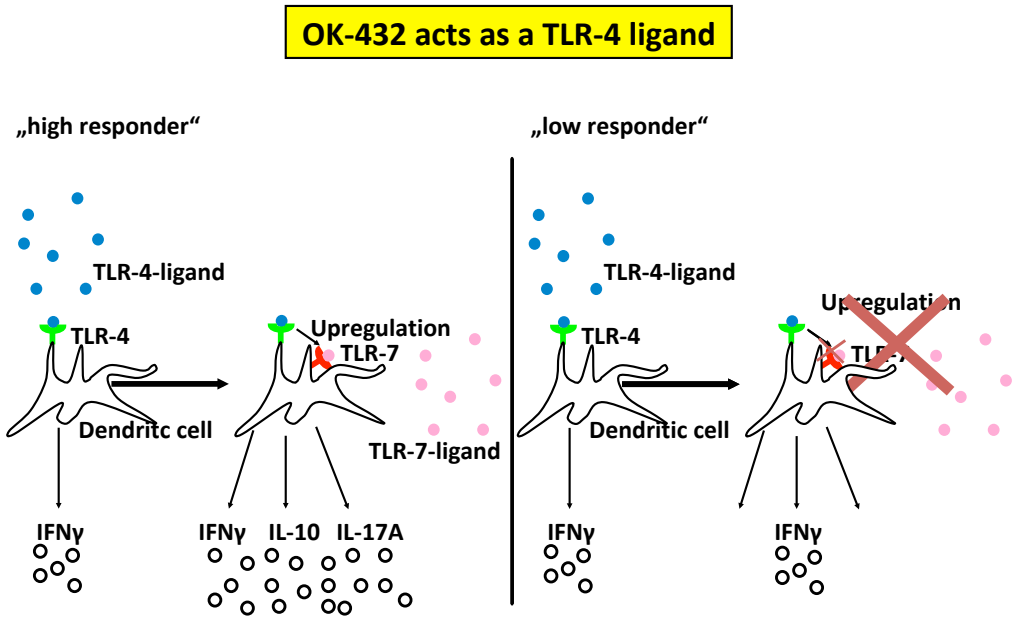


Fig 12 Illustration of hypothesis in Paper I and II. TLR4-dependent up-regulation of TLR7 in monocytes of HRs leads to extensive immune reaction. Monocytes of LRs show a reduced immune reaction with limited cytokine production due to deficient TLR7 expression.

Several authors have shown evidence that OK-432 sclerotherapy is a safe and effective method and it has been suggested to be the first line treatment for LMs [45, 51, 62].

The long-term clinical outcome of patients treated with OK-432 has not been well described in large cohorts[58, 63, 64].

The success rate after treatment of LMs with OK-432 varies from 40% to 80% and cannot be predicted. Even in the case of macrocystic disease where one expects a successful outcome, every fifth patient is treated unsuccessfully. Success is defined as size reduction by inflammatory shrinkage by at least 50 %. Other factors than the type of LM must be involved. Furthermore, the use of sclerosing agents sometimes causes unpredictable scarring due to the penetration of adjacent tissues, to the extent that subsequent surgery is difficult or impossible. [40, 42, 45, 50, 58, 65, 66]. Prenatal anatomical characteristics may predict outcome.

In spite of being feasible to treat LMs with OK-432 the patients show variable response to the treatment and occasionally the treatment is followed by significant swelling that can compromise vital functions such as the breathing. To what extend swelling follows the treatment is difficult to predict. The type of LM, microcystic, macrocystic, or mixed lesions, is often considered to be a prognostic factor for the outcome of sclerotherapy[67, 68].

Different outcomes in patients with the same type of lesion may indicate that individual factors have an influence on the response to the treatment. One explanation for the variation in clinical response to OK-432 treatment may be individual immune- pharmacological prerequisites[57, 58].

Mediastinal LMs are of special concern and are prone to severe complications after sclerotherapy. Patients show variable response to OK-432 and occasionally the treatment is followed by significant swelling that can compromise vital functions such as the breathing. Lesions in the mediastinum represent a special challenge in this sense due to the narrow compartment with vital structures. A multidisciplinary team should always tailor the treatment of LMs for each patient individually and the potential risks for each treatment modality must be considered[48, 49].

2.5 OUTCOME MEASURES

The clinical presentation and symptoms of LMs depend on type, size and location. The indications for treatment are individualized and differ within the group without clear treatment protocols making systematic assessment of outcomes challenging or impossible. A systematic meta-analysis on intra-lesional bleomycin injections for vascular malformations revealed that most authors use the size of the malformation and the shrinkage as the primary outcome measure[69]. Size is best evaluated with imaging techniques. To determine the exact shrinkage of the malformation after treatment imaging should be performed with the same modality as prior to the treatment. If the radiological work-up for LMs is done with MRI, the follow-up should be done with MRI. This is of course not practical as MRI often is an invasive investigation within the pediatric population, as it in many occasions requires fully anesthetized children and it is also expensive. Therefore, clinicians often estimate size and volume of the malformations prior and after treatments by subjective non-validated methods. Other outcome measures such as symptom relief, patient satisfaction or quality of life are difficult to find in the literature.

Many therapeutic studies have been published, but the heterogeneity in outcome measures used to determine treatment success or failure makes it difficult to compare or combine results[70, 71].

There is a need for standardization of outcome measurement in clinical research studies of vascular malformations. Recently an international multicenter consensus study, (the Outcome measures for VAscular MAlformations project, OVAMA) suggested core outcome sets for clinical research on peripheral vascular malformations, including Lymphatic malformations (LM), Venous Malformations (VM) and ArterioVenous Malformations (AVM). In order to achieve that, an expert panel along with patients and parents agreed on what the most important outcome measure domains are for the largest subgroups of vascular malformations and *how* they should be measured. The core outcome measures for LM that were agreed on was the overall health related quality of life (HRQOL), the activity of daily living (ADL), recurrence, radiological assessment, overall severity of symptoms, pain, location specific signs and infections[72].

HRQOL refers to the subset of quality of life directly related to an individual's health, which as defined by the World Health Organization includes physical, mental, and social wellbeing.

It has become an increasingly important outcome measure from the clinical and epidemiological point of view[73, 74].

This development is a result of the understanding that ¨cure¨ is an insufficient goal of care, as optimal health should include physical, psychological, social and cultural dimensions of wellbeing and functioning[75].

Childhood illness, severe or chronic, may potentially affect quality of life. Therefore, increased attention is being paid to the impact of consequences of illness and treatment on quality of life of patients[76].

Studies have showed that patients with rare diseases, such as vascular anomalies, despite treatment outcome, have an increased risk of physical and psychological undesirable long-term consequences[77-80].

HRQOL is sparsely studied in the literature in patients treated for LMs. In a Finnish study of long-term follow up after treatment of head/neck or trunk LMs, a subset of 26 patients were subject to an explorative quality of life survey. It was concluded that a majority of the patients did well after sclerotherapy[81].

The Clavien-Dindo classification is widely used to evaluate surgical complications and objectively grades complications after surgery in relation to the extent of treatment required to cope with the complications[82]. The Clavien-Dindo classification has previously been used for a systematic evaluation of complications after sclerotherapy on venous malformations[83].

In order to bring forward a treatment algorithm for patients with LMs it is essential to both measure and evaluate the clinical outcomes as well as assessing the complications to the various treatment using the same evaluation tool despite if the patients have been operated or received sclerotherapy. Clavien-Dindo classification to stratify the postoperative complications is widely used in surgery and provides an objective measure of the complications on a scale based on the severity[82].

The need of antibiotics, pain relief, parenteral nutrition and blood transfusion are considered to be grade I-II complications. Grade III-IV complications are complications requiring surgery or radiological intervention and need of intensive care in general anesthesia. The Clavien-Dindo classification has been successfully used for a systematic evaluation of complications after sclerotherapy on venous malformations[83].

The various treatment modalities for LM especially sclerotherapy may be evaluated concerning complications using Clavien-Dindo classification in order to be objectified and comparable.

3 AIMS

Aims of the thesis

The main objective of this project was to evaluate the outcomes of treatment of LMs, identify prognostic factors and better understand the inflammatory mechanisms of OK-432 treatment.

Aims of the specific studies

1. To evaluate TLR4 expression as a tool to predict the outcome of OK-432 injection treatment in order to improve selection of patients for sclerotherapy (Paper I).

2. To further investigate the immunological response of monocytes after LPS stimulation to understand why patients respond differently in spite of having a similar malformation (Paper II)**.**

3. To describe the demographics and long-term outcomes of a large cohort of patients with LMs managed with OK-432 sclerotherapy (Paper III).

4. To evaluate predictive factors for the outcome of OK-432 sclerotherapy (Paper III).

5. To review the management and outcomes of patients with LMs involving the mediastinum and to propose a treatment algorithm to guide the management of these rare malformations (Paper IV).

6. To assess the complications after sclerotherapy as well as surgery for LM with the Clavien-Dindo classification (Paper IV).

7. To assess the long-term HRQOL in a cohort of Swedish children and adolescents with LMs treated with sclerotherapy (Paper V).

4 SUMMARY OF THE STUDIES

4.1 PATIENT AND METHODS

Analysis of TLR expression on monocytes (Paper I and II)

In Paper I and II we analyzed blood from eleven children with LMs. All children had been treated with OK-432. Five of the children had a good result (high responder, HR, n =5) of the treatment and six children insufficient result (low responder, LR, n =6) after treatment with OK-432. All children were assessed with clinical evaluation and with ultrasound to measure the size of the malformation. A good result was defined as shrinkage of >90% of the original size of the whole malformation. An insufficient result was shrinkage by <50%, which was considered to ensure a sufficient difference between the groups. Only patients with doubtless results were included in order to distinguish the groups clearly. The groups were established before the laboratory analysis and no change was performed thereafter. The blood samples were coded and a blinded analysis was performed.

After harvesting the monocytes with pipettes the cells were suspended with 1% penicillin/streptomycin and each sample were split up in two fractions and incubated on at human body temperature. One of the two fractions was incubated with the TLR-4 ligand, Lipo-Poly-Saccharide (LPS) for 20h. OK-432 is acting as a TLR-4 ligand but represents a combination of different substances with effects that are not fully defined. It could therefore not be used to selectively investigate effects of TLR4 binding.

After the incubation time, the cells were harvested again and stained with antibody staining for identification of cells (monocytes), receptors (TLR) on the surface (TLR4) and intracellular (TLR7) identification. The isotype controls were treated exactly like the other samples.

We used Fluorescence-activated cell sorting (FACS) (Fig 13) before and after LPS stimulation of the monocytes to detect the presence of TLR4 and TLR7 receptors on the surface of the cells. FACS is a useful scientific instrument as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest.

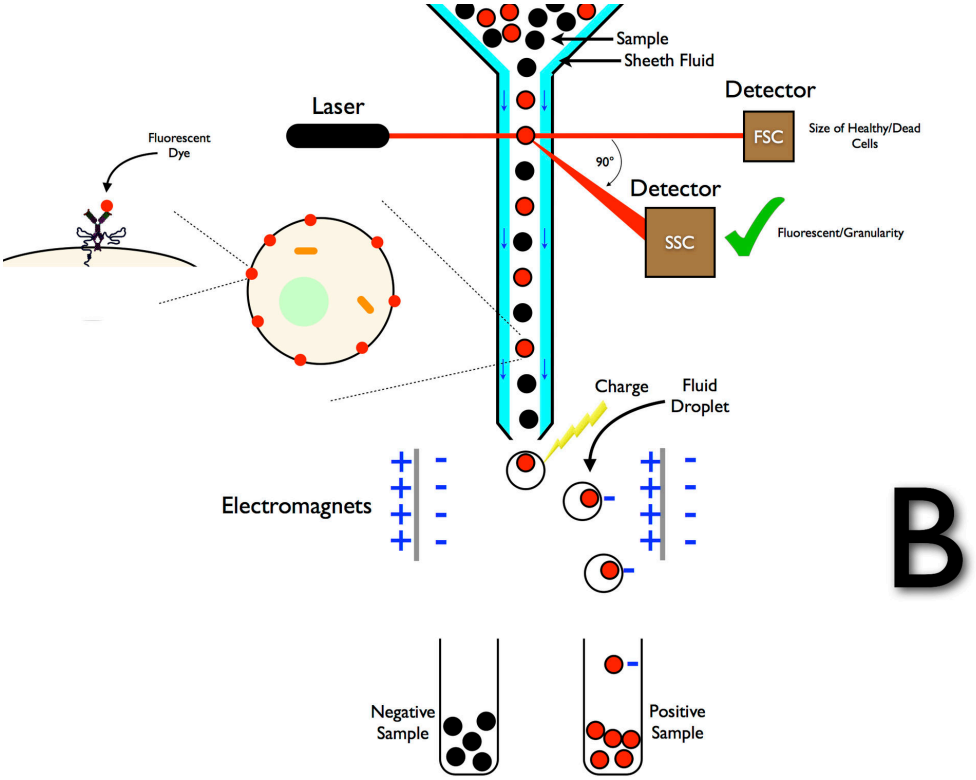


Fig 13 FACS, TLR4 and 7 specific Fluorescence dye is used to stain the receptors on the surface of the cells. The fluorescence activity on the surface of the cells is measured with laser and the cells are also sorted based on the respective electromagnetic gradient[84].

In Paper I we hypothesized that the immunological mechanism of OK-432 treatment was conducted through stimulation of TLR4 on the surface of the monocytes that will lead to an intracellular up-regulation of TLR7 and thus a much stronger inflammatory response on HR patients compared with LR patients.

In Paper II we further analyzed the mean fluorescence intensity (MFI) and corrected by the fluorescence of the isotype controls (corrected MFI) (Fig 14).

The absolute expressions of TLR4 in the HR and LR cells with and without LPS stimulation were investigated.

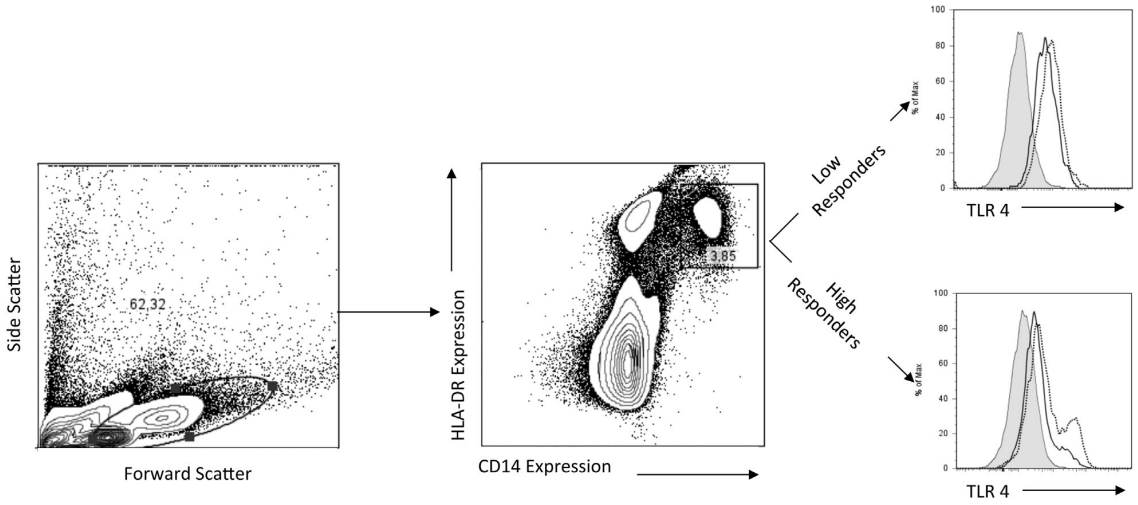


Fig 14 Gating protocol for identification of HLA-DR^{D}CD14^{D} monocytes. Analysis of TLR4 expression on monocytes in high- and low-responding patients (mean fluorescence intensity; gray filling, isotype control; continuous line, nonstimulated control; dotted line, sample after stimulation with LPS).

Patient demographics and clinical assessment (Paper III)

Paper III was a cross-sectional, observational study of 138 patients with LMs treated with OK-432 at the Astrid Lindgren Children´s Hospital, between 1998 and 2013. The final group for the study after exclusion due to loss to follow up consisted of 131 patients; 74 females and 57 males. The malformations were categorized according to the ISSVA classification (International Society for the Study of Vascular Anomalies)[23]. All patients received OK-432 (0.1 mg, in 10 ml NaCl solution) and the injection treatment was guided with ultrasonography. Injection therapy was repeated if the outcome was considered to be unsatisfactory at follow-up. The charts were retrospectively reviewed. The outcomes were assessed with clinical examination and a questionnaire. MRI is a well-recognized method for evaluating patients with LM after sclerotherapy. However, MRI is an invasive examination in pediatric patients, due to the need for general anesthesia, and it is also expensive. We chose to evaluate the patients clinically, using a clinical assessment scale (CAS) as a follow-up instrument (Table 1). The clinical outcome of the patients was assessed with the clinical assessment scale (CAS) that was tailored for clinical evaluation in Paper III. The last MRI was analyzed for postoperative or postsclerotherapy evidence of remaining LM in the mediastinum.

Clinical evaluation	Result	Score
No remaining signs and symptoms	Excellent	5
Small palpable lumps or regress of mass > 50%, rarely symptomatic	Good	4
Larger palpable lumps or regress of mass < 50%, occasionally symptomatic	Fair	3
No regress of signs and symptoms	Poor	2
Worsening of signs and symptoms	Worse	1

Table 1 Clinical Assessment Scale (CAS) for clinical evaluation of LM

Patient or parent satisfaction was estimated based on the questionnaire. The patient satisfaction scale in the questionnaire comprised a score of five for very satisfied, four for satisfied, three for acceptable, two for unsatisfied and one for very unsatisfied. The impact of 11 variables on the clinical outcome was tested; *age at symptom debut, time to confirmation of diagnosis, gender, lesion location, side, lesion type, estimated LM volume previous treatment with either resection or another type of sclerotherapy, number of OK-432 injection treatments, age at first treatment and more or less than five years of follow-up*. These variables were chosen since they were systematically collectable in our records and also because we considered them to possibly affect HRQOL.

Assessment of complications of sclerotherapy and surgery (Paper IV)

In order to bring forward a treatment algorithm for patients with LM involving the mediastinum we needed to measure the clinical outcome and assess the complications of this group of patients using same evaluation tools both for the patients that have been operated as well as those who had received sclerotherapy. This was a cross-sectional, observational study. The charts were retrospectively reviewed of all patients with LMs involving the mediastinum treated at our institution between 2009 and 2015. We collected

demographic data; data on investigations, management, and complications of treatment, as well as outcomes at follow up. We used the Clavien-Dindo classification to stratify complications (Table 2). The need of antibiotics, pain relief, parenteral nutrition and blood transfusion was considered to be grade I-II complications. Grade III-IV complications were complications requiring surgery or radiological intervention and need for intensive care in general anesthesia. The need for mechanical ventilation more than eight days in the PICU after sclerotherapy was considered grade III complication. The use of repeated sclerotherapy, without symptomatic relief in an intensive care patient, was considered a grade III complications and comparable with other radiological interventions. Each complication was counted in order to quantify complications per treatment.

Grade	Clavien-Dindo Classification of Surgical Complications
1	Any deviation from the normal postoperative course without the need for pharmacological treatment or surgical, endoscopic, and radiological interventions. Allowed therapeutic regimens are: drugs such as antiemetics, antipyretics, analgetics, diuretics, electrolytes, and physiotherapy. This grade also includes wound infections opened at the bedside.
2	Requiring pharmacological treatment with drugs other than allowed for grade 1 complications. Blood transfusions and total parenteral nutrition are also included.
3a	Surgical, endoscopic, or radiological intervention without general anesthesia.
3b	Surgical, endoscopic, or radiological intervention with general anesthesia.
4a	Life-threatening complication requiring intermediate care or intensive care unit management, single organ dysfunction.
4b	Life-threatening complication requiring intermediate care or intensive care unit management, multi-organ dysfunction.
5	Death of a patient.

Table 2 The Clavien-Dindo classification of surgical complications[82]

Assessment of HRQOL in patients with LM (Paper V)

All patients who underwent injection treatment with OK-432 for LMs at Karolinska University Hospital in Stockholm, Sweden, between 1998 and 2013 were retrospectively reviewed. The LMs were diagnosed by physical examination, ultrasonography and magnetic resonance imaging. Malformations were classified according to the International Society for the Study of Vascular Anomalies (ISSVA) classification based on the radiological appearance in macrocystic, microcystic or mixed LMs. The classification does not take the size of the malformation or the number of cysts into account. The lesions were also categorized according to the anatomical localization[85].

A questionnaire was sent to all patients with at least five years' follow up after the first injection treatment asking for persisting symptoms and satisfaction with the treatment and care. The KIDSCREEN-52 was used to assess HRQOL[73]. KIDSCREEN is a standardized tool for assessing children´s and adolescents´ subjective health and wellbeing[74].

The KIDSCREEN was developed as a European cross-national cooperation providing validated data from 13 European countries. The dimensions covered by KIDSCREEN-52 are *Physical Well-being, Psychological Well-being, Mood and Emotions, Self-perception, Autonomy, Parent Relations and Home life, Financial Resources, Social Support and Peers, School environment, and Bullying.*

The European validated general population norm data for KIDSCREEN cover the 8 to 18 years' age range. Swedish validated norm data cover the 12-18 years span only. In this study we therefore used the European norm data as the reference for the entire group, while in addition Swedish norm values were used as the reference for the subgroup of 12-18 years old patients.

A comprehensive study-specific questionnaire was also developed for this project. Information from that questionnaire was derived regarding patient-reported occurrence of persistent visible signs of illness/treatment (yes/no), and self-reported patient satisfaction with treatment (along a 5-grade Likert scale ranging from *very satisfied* to *very dissatisfied*). The treatment outcome was categorized on a six-step scale based on remaining signs of the malformation assessed on a clinical evaluation ranging from *excellent* to *worse*. For the statistical analysis the variable was dichotomized into 1 (the two best categories), and 2 (the four poorest categories).

Statistics

Paper I-II

Expressions of the TLRs in monocytes after stimulation with LPS were compared with expressions in nonstimulated cells (response ratio). Two tailed unpaired t-test statistical analysis was performed with GraphPad Prism software (version 5.04, GraphPad Software, La Jolla, CA). The values are shown as mean ± standard deviation (SD) or standard error of the mean (SEM). $P< 0.05$ was considered significant.

Paper III-IV

Data are presented as frequencies or median (range). Data were analyzed by a nonparametric one-way analysis of variance. Spearman's rank order correlation test was used to assess for concordance between the first and second clinical assessments. The Mann–Whitney U-test was used to compare groups. The statistical analysis was performed using a statistical software package Statistica (Statistica 10; StatSoft Inc, Tulsa, OK, USA). $P <0.05$ was considered statistically significant.

Paper V

Descriptive statistics were provided for patient-reported occurrence of persistent visible signs, and satisfaction with treatment, and for the interrelatedness between these variables and the HRQOL dimensions. Continuous variables were presented with median and inter-quartile-range, and categorical variables were presented as frequencies and percentage.

HRQOL in the patient group was compared to reference values in Sweden and Europe. One-sample t-test was implemented to compare the patient groups T-values of HRQOL domains to the reference population mean for each domain.

The impact on HRQOL was measured regarding four continuous variables; *age, volume of the malformation, total number of treatments, and annual number of treatments,* as well as five categorical background variables; *localization of LM, result of treatment, type of LM, and gender.* Localization of LM was categorized into two groups; head/neck and other localization, results of treatment were categorized into two groups. Group 1, the two best categories, and group 2 the four poorest categories. The impact of each variable on the HRQOL domain was assessed using ANOVA F-test. Multiple models were not considered.

The estimated strength of association between continuous predictors and HRQOL domains was presented with Pearson correlation coefficient. Statistical significance was set to $p<0.05$. All analysis was run in R version 3.3.2.[86]

4.2 RESULTS

Expression of TLR4 on monocytes after cell stimulation (Paper I and II)

In the group of patients with good treatment result (HR), with OK-432 there were two boys and three girls and in the group with insufficient result (LR) there were two boys and four girls. All patients had clinically large malformations with significant psychosocial impact. The total size of the malformations and the size of the cysts within the malformation that categorizes the type of LM were comparable between the groups. The mean age in the HR group was 1.4 ± 0.9 (range 0.5-2.6) y and 2.8 ± 2.9 (range 0.7-7.4) p=0.31. The analysis was performed after a mean period of 44 months in the HR group and 49 months in the LR group respectively after the end of the treatment. There was no statistical difference between the groups. The hypothesis could not be verified with respect to TLR7 expression. However TLR4 expression was significantly increased in the group of patients with high response after OK-432 treatment. We did additionally observe that malformations with similar morphology responded differently after TLR4 stimulation with LPS.
In **Paper I** intracellular staining for TLR7 expression revealed a slight mean increase by a factor 0.5 in the HR group and a mild decrease by the factor 0.6 in the LR group, respectively after LPS stimulation compared with the unstimulated isotype controls. The difference did not reach statistic significance. However the expression analysis of TLR4 showed a relative increase after LPS stimulation in the HR group by the factor 3.6, which was significantly higher than in the LR group (factor 1 compared with nonstimulated isotype controls, P=0.037).

In **Paper II** prestimulation values of TLR4 expression in the LR group compared with the HR group were (950 ± 718 vs. 477 ± 341). Mean MFI after LPS stimulation was comparable in both groups (HR 1142 ± 652 units, LR 839 ± 427 units; mean value ± SD; P = 0.85). This observation is in accordance with the analysis of the mean absolute differences between TLR4 expressions in cells of samples with and without LPS stimulation (HR 665 ± 683 vs. LR -111 ± 605; P = 0.08), showing mean down-regulation in the LR group.

Characteristics of patients with LM and clinical outcome (Paper III)

Patient characteristics are outlined in Table 3 and compared with data from a similar study. Diagnosis was prenatal in five (4%) patients and neonatal in 54 (42%) patients, with 17 (13%) diagnosed during infancy, 29 (23%) at one to three years of age and 23 (18%) after three years of age. External malformations that expanded into the mediastinum and, or, the retroperitoneal cavity were encountered in eight (6%) patients. Estimated median volume of the lesions was 35ml (range 2-1500). Time from debut of symptoms to confirmation of the LM diagnosis was 1.2 years (range 0-21).

	Present study	Burrows et al (ref)
	n= 131	n=41
Age, median (range)	3.4y (2 m-78 y)	6.9y (3 m -31 y)
F/M	80/58(58/42)	24/17(59/41)
Location of lesion		
-Head-neck	79(60)	27(66)
-Truncal	26(20)	8(19)
-Extremities	8 (6)	6(15)
-Combined	18(14)	0 (0)
Lesion type		
-MacC (>1 cm)	27(21)	20(49)
-MicC (<1cm)	13(10)	3(7)
-Mixed	89(69)	18(44)
Prior surgical excision		
	5(4)	18(44)

Table 3 Baseline characteristics of this study compared with Burrows et al ref. Abbreviations: y=years, m=months, MacC=macrocystic, MicC=microcystic, Mixed=mixed type.(%)

After OK-432 treatment, symptoms such as local swelling, low-grade fever and redness were observed in the vast majority of the patients, but were regarded as expected side effects of the treatment.

Major adverse events such as re-admission to hospital and, or, emergency surgery due to major swelling affecting vital functions occurred in five of the patients. All of them had

mediastinal involvement of the malformation. Delayed extubation (8-17 days) due to airway compromise were planned in 15 patients and they were observed post-treatment in the intensive care unit. Protective pre-treatment tracheostomy was given to five patients and two additional patients received a tracheostomy after repeated failed attempts to wean them off mechanical ventilation. All these patients had large cervical or intra-thoracic lesions. We did not observe any case of ulceration, scarring, nerve damage or persistent swelling due to the OK-432 treatment. In total, 19 patients received surgery, three primarily and 16 after unsatisfactory results of OK-432 treatment. Unsatisfactory results of OK-432 treatment were considered after a median number of six injection treatments (range 1-11). Of the primarily operated patients, two received surgery because of the risk of compromise of the airways. Unsatisfactory results from sclerotherapy were the cause for the operation in 12 patients with LM involvement of the head/neck region, one on the trunk and three lesions on multiple regions. The type of malformation that required an operation due to unsatisfactory results from sclerotherapy was microcystic in four cases, macrocystic in one case and mixed lesions in 11 cases. The patient with a macrocytic malformation was a newborn girl with a huge extra thoracic LM. She underwent a failed attempt of intra-lesional sclerotherapy with ethanol. Her general condition improved and she was accepted for OK-432 sclerotherapy. However, that treatment also failed and she was successfully operated on.

The CAS was worse in one patient, poor in 14, fair in 24, good in 39 and excellent in 53 patients. The age at first treatment with OK-432 was 3.4 years (0.03-75) and the median time to last follow up after the first injection treatment was nine years (1-19). Patients with microcystic malformations showed excellent or good results in five out of 13 cases and all of them had LM volumes less than 15 cm^3 (Table 4).

	Number of treatments, median (range)	Worse	Poor	Fair	Good	Excellent	n
MicC	3 (1-9)	0	4	4	3	2	13
MacC	2 (1-7)	0	1	1	7	18	27
Mixed	2 (1-12)	1	9	19	29	33	91
Total	2 (1-12)	1	14	24	39	53	131

Table 4 Lesion type, number of injections, and outcome. Abbreviations: MicC= microcystic, MacC=macrocystic, Mixed=mixed type

Previous OK-432 treatment, resection and, or, another type of sclerotherapy, as well as localization to head and neck predicted a less favorable outcome (p=0.008 and p=0.016, respectively). Furthermore, four or more injection treatments also predicted a less favorable outcome compared to those treated with fewer injections (p=0.0003). The 29 patients who were assessed twice had similar outcomes at both assessments, the first in 2002 and the second in 2013.

Mediastinal LM, clinical outcome and complications (Paper IV)

Between 2009 and 2015 we treated seven patients, five girls and two boys, with LMs expanding into the mediastinum. Patient characteristics are summarized in Table 5 and complications and outcome is summarizes in Table 6.

	Pat 1	Pat 2	Pat 3	Pat 4	Pat 5	Pat 6	Pat 7
Pre-natal diagnosis	Y	N	Y	N	Y	N	N
Neo-natal diagnosis	N	N	N	Y	N	Y	Y
Late diagnosis	N	Y	N	N	N	N	N
Full term	Y	Y	Y	Y	N	Y	Y
EXIT-delivery	Y	N	N	N	N	N	N
Type of LM	Mixed	Mixed	Mixed	Mixed	Mixed	Mixed	Mixed
Cervical components of the malformation	Y	N	Y	Y	N	Y	Y
Respiratory distress at time of diagnosis	Y	Y	Y	N	Y	Y	N
Anatomic airway compromise at time of diagnosis	Y Lung	Y Distal trachea	Y Distal trachea	N	Y Lung	Y Distal trachea	N
Circulation compromise at time of diagnosis	N	N	N	N	Y	N	N
Primary sclerotherapy	N	Y OK-432 0,1 mg x1	Y Sotradecol 3% 20 mL x1	N	Y Doxycyclin 50 mg x9	Y OK-432 0,1 mg x4	Y Bleomycin 6000 IE x3

Table 5 Patient characteristics. Abbreviations: Yes,Y; No,N

	Pat 1	Pat 2	Pat 3	Pat 4	Pat 5	Pat 6	Pat 7
No of complications grade 1-2 * After primary sclerotherapy	Prim surgery	3	4	Prim surgery	4	5	3
No of complications Grade 3-5 * after primary sclerotherapy	Prim surgery	6	7	Prim surgery	11	12	6
Days in ICU after primary sclerotherapy		31	14		*	9	10
Number of surgical procedures	1	2	2	1	2	2	0
No of complications grade 1-2 * After surgery	4	4	4	4	4	Only cervical excision	Only sclero
No of complications grade 3-5 * after surgery	3	2	1	1	1	Only cervical excision	Only sclero
Days in ICU after surgery	Trach ***	5	8	3	**	Only cervical excision	Only sclero
Time after surgery/ sclero at last follow-up	4,25 y	3,17 y	1,33 y	2,92 y	4,17 y	6,42 y	2,25 y
Clinical assessment score (1-5)	5	5	5	5	5	3	4
LM in mediastinum on MRI at last follow-up	Y	N	N	N	N	Y	Y

Table 6 Complications and outcome * Complications according to Clavien-Dindo classification** Premature infant, long NICU time due to prematurity, *** Infant with pulmonary hypoplasia requiring long time ventilation support

The vast majority of the patients (71%) had respiratory distress when the diagnosis was confirmed at postnatal MRI. All patients had predominately macro-cystic LMs with part of the malformations containing micro-cystic components, being classified as mixed LMs. Airway compromise at the time of diagnosis was common, and was encountered in 57% of the patients. Three patients had anatomical compression of the trachea and two patients had sub-total compression of the right lung. All three patients with tracheoscopy-verified compression to the trachea had compromise of the distal trachea, and a tracheostomy would not have been protective. Only one patient was given a pre-treatment protective tracheostomy and one patient required a post-operative tracheostomy due to prolonged need of mechanical ventilation.

All patients received sclerotherapy. Five patients were treated with sclerosing agents as first line of treatment and two had sclerotherapy as an adjunctive intra- and postoperatively. The sclerosing agents and doses used were OK-432 0,1 mg in four patients and bleomycin 6000 IE, doxycycline 50 mg, and sotradecol 3% 6,5 mL in one patient respectively. Two of the patients that were treated with sclerotherapy, were observed at the pediatric intensive care unit, PICU, and on mechanical ventilation, until the maximum swelling phase of the treatment started to decline. Shortly after discharge, both patients were re-admitted to the hospital and to the PICU, due to acute airway obstruction and mediastinal deviation leading to compromise of the intra-thoracic circulation. A third patient received a protective tracheostomy before sclerotherapy. The tracheostomy did not prevent obstruction on the trachea distal to the stoma. The patients treated with sclerotherapy experienced in median four (range 2-6) Clavien-Dindo grade I-II complications and in median seven (range 6-12) Clavien-Dindo grade III-IV complications. The median time with mechanical ventilation at the neonatal intensive care unit (NICU) after each sclerotherapy was eleven days (range 8-31). There was no difference in post-treatment need of mechanical ventilation and observation at the NICU between the different sclerosing agents. Patient 1 and patient 5 are excluded from these figures since they were intubated directly after birth and could not be weaned off mechanical ventilation before they were operated.

Five of the patients (71%) were operated with excision of the LM in the mediastinum. The operative procedure was done accessing the mediastinum through a median sternotomy. The malformation was excised/ de-bulked without sacrifice of vital structures. Intra-operative sclerotherapy was administrated to all patients. Intraoperative sclerotherapy after excision of the bulk of the malformation was provided to the proximity of the malformation and the

excised margins to deeper lymphatic ducts. Two of the patients were operated primarily and three patients were operated after life threatening complications to sclerotherapy. The operated patients experienced in median three (range 2-4) Clavien-Dindo grade I-II complications and in median two (range 1-4) complications of Clavien-Dindo grade III-IV. No patients experienced function loosing surgical complications such as nerve injury or vocal cord palsy. One patient required extended post-operative neonatal care at the neonatal intensive care unit, NICU, due to prematurity before she could be discharged from the hospital, and one patient received a postoperative tracheostomy as she was in need of a prolonged ventilatory support due to suspected pulmonary hypoplasia. The remaining three patients were operated and were discharged from the Pediatric Intensive Care Unit (PICU) after 3, 5, 8 days respectively. No patients were readmitted to the PICU for ventilator support after discharge.

The mean follow-up time was 3,5 years (range 1,3-6,4). The clinical assessment at last follow up was excellent for 5/7 patients. Two patients that only received sclerotherapy were assessed as good and fair respectively.

HRQOL in patients with LM treated with OK-432 (Paper V)

One hundred and thirty-eight patients had been treated with OK-432 for LM from 1998-2013. In 40 patients the follow up from first injection treatment was less than 5 years. Thirty-nine patients were less than 8 years of age or older than 18 years of age at follow up. Forty-nine of 59 (83%) patients who were eligible for the study responded to the questionnaires. Demographics and background data of the 49 included patients are presented in Table 7.

n	49
Gender=male (%)	18 (36.7)
Age at assessment (years, mean (sd))	12.41 (2.87)
Time from first treatment to assessment (years, median [IQR])	8.94 [6.84, 11.38]
Volume of the malformation (ml, median [IQR])	40 [20,100]
Total number of injection treatments (n, median [IQR])	3 [2,5]
Annual number of injection treatments (n, median [IQR])	0.18 [0.11,0.34]
Localization, n (%)	
not head-neck	16 (32.7)
head and neck	33 (67.3)
Type of malformation, n (%)	
macrocystic	14 (28.6)
mixed LM	30 (61.2)
microcystic LM	5 (10.2)
Patient satisfaction, n (%)	
satisfied	36 (73.5)
not satisfied	13 (26.5)

Table 7 Patient demographics and background data

The results for the KIDSCREEN-52 are shown as T-values in Table 2A and Table 2B. Swedish patients treated with sclerotherapy for LM scored higher than the European reference values for *Autonomy* (p=0.014), *Financial Resources* (p=0.015)*, School Environment* (p=0.004) *and Bullying* (p=0.041). Regarding the other KIDSCREEN-52 dimensions LM patients did not differ from the European reference values (Table 2A). No differences were found between 12-18 years old LM patients and Swedish norm values (Table 8).

	Patient group			Norm values SE		
Dimensions	**Mean**	**SD**	**n**	**Mean**	**SD**	**p-value**
Physical Well-being	48.9	8.88	29	48.0	8.88	0.576
Psychological Well-being	48.7	9.94	29	49.5	9.94	0.647
Moods & Emotions	49.7	11.55	29	49.8	11.55	0.923
Self-Perception	46.6	9.11	29	49.1	9.11	0.142
Autonomy	53.4	10.67	29	50.8	10.67	0.196
Parent Relation & Home Life	51.9	9.06	29	52.2	9.06	0.858
Financial Resources	54.8	7.92	29	52.3	7.92	0.097
Social Support & Peers	52.3	11.67	29	51.7	11.67	0.794
School Environment	53.0	12.43	29	51.3	12.43	0.474
Bullying	53.7	8.21	29	52.9	8.21	0.623

Table 8. LM patients, 12-18 years of age, vs Swedish norm values (T-values) for KIDSCREEN-52, 12-18 years.

The relationship between the continuous variables and the studied HRQOL dimensions are shown in Table 9. Statistically significant negative correlations were found between the total number of injection treatments and *Autonomy* (p=0.013), *Parent Relation and Home Life* (p=0.014), *Financial Resources* (p=0.025), *and School Environment* (p=0.037).

	Correlation				p-values[a]			
	Age	**Volume**	**No of inj**	**Inj./year**	**Age**	**Volume**	**No of inj**	**Inj./year**
Physical Well-being	-0.13	0.18	-0.25	-0.16	0.379	0.234	0.090	0.271
Psychological Well-being	-0.21	0.21	-0.18	-0.08	0.143	0.146	0.212	0.612
Moods & Emotions	-0.29	0.00	-0.24	-0.12	0.044	0.983	0.100	0.424
Self-Perception	-0.39	-0.12	-0.07	0.03	0.005	0.406	0.648	0.845
Autonomy	-0.03	-0.21	-0.36	-0.26	0.823	0.145	0.013	0.073
Parent Relation & Home Life	-0.09	-0.08	-0.35	-0.23	0.526	0.569	0.014	0.121
Financial Resources	0.25	0.21	-0.33	-0.29	0.097	0.166	0.025	0.056
Social Support & Peers	-0.10	0.07	-0.26	-0.18	0.504	0.644	0.077	0.225
School Environment	-0.29	0.08	-0.31	-0.17	0.050	0.586	0.037	0.256
Bullying	0.12	0.08	-0.09	-0.07	0.420	0.583	0.562	0.666

[a] F-test, two-tailed.

Table 9 Associations between HRQOL dimensions and age, volume, total number of injections, and injections per year

Outcomes for the categorical variables are shown in Figure 1. Localization in the head neck area was a negative predictor throughout all studied dimensions with the strongest correlation with *Psychological Well-being* (p=0.009), *Parent Relation and Home Life* (p=0.017) and *School Environment* (p=0.006). Less satisfying result of the treatment was also noticed as a negative predictor for all HRQOL dimensions (Fig 15). Gender was not a significant

predicting variable except for girls in the dimension *Mood and Emotions* (p=0.034) in combination with head-neck localization of the malformation.

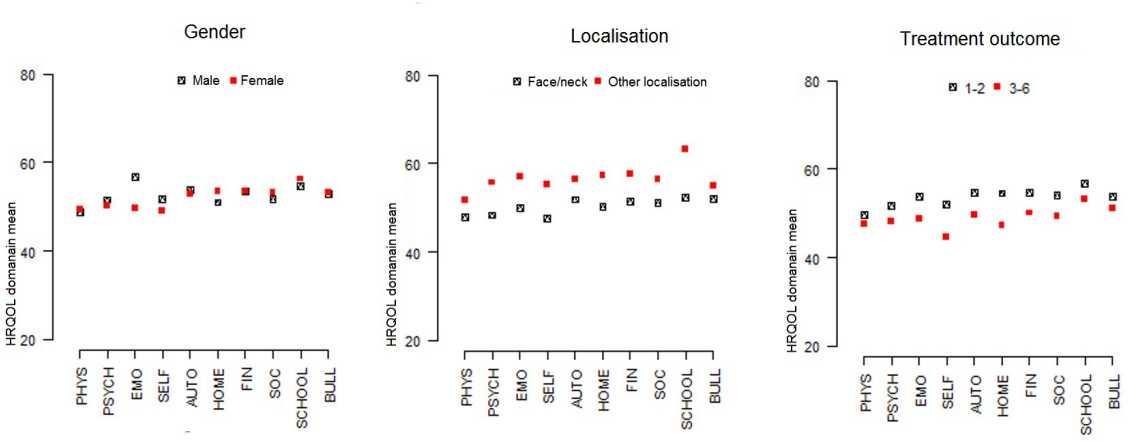


Fig 15 Plot charts for associations between HRQOL dimensions and gender, localization and treatment outcome ("1-2" = the two best treatment outcome categories; "3-6" = the four poorest treatment outcome categories).

Visible signs of the LM were persistent in 44.9% of the patients. Bivariate analysis indicated that persistent visible signs *per se* were unrelated to HRQOL outcomes. However, patients with visible signs were less satisfied with treatment (Fishers Exact Test, p = <0,001). All but one, .i.e., 96% of patients with no visible signs were very satisfied with treatment. The corresponding portion in patients with persistent signs was 29%. Also, the degree of experienced overall *difficulty* of remaining problems including signs of illness was negatively correlated with satisfaction with treatment (r= -0.384, p = 0.011).

4.3 DISCUSSION

Dynamic Toll-like receptor expression predicts outcome of sclerotherapy for lymphatic malformations with OK-432 in children (Paper I and II)

The hypothesis that expression of TLR7 would predict outcome of OK-432 treatment could not be verified since there were no significant differences between the groups after LPS stimulation. This could be explained by the fact that LPS is a selective TLR4-ligand and is not primarily directed against TLR7. TLR4 expression was significantly increased in the group of patients with high response after OK-432 treatment. This result is consistent with the expected enhanced TLR4- dependent immunologic effect in this group compared with the LR group. Differential responses to LPS in healthy humans on the basis of common TLR4 variants have been described[87, 88].

It remains elusive if these mechanisms play a role in the present patients. According to clinical measures, however, the malformations were comparable. A drawback is the analysis of TLR expression after treatment with OK-432. Although the mean period between treatment and analysis is at least 44 months in both the groups, an influence of the immunostimulatory treatment on the result cannot be fully excluded. A possible influence can only be ruled out with a prospective study that gives a chance for an analysis before the treatment. We could identify TLR4 as a candidate parameter, which most probably can be used to predict the outcome of treatment of LMs with OK-432. However, the observation of relative TLR4 expression changes could be explained by two different conditions: either a substantial increase of TLR4 expression after LPS treatment or differences of expression before treatment with similar expression patterns after incubation with LPS. We showed comparably high TLR4 expression values in monocytes of patients with low treatment results without relevant differences after LPS stimulation. Treatment results can be possibly be predicted on the basis of absolute values before LPS stimulation.

Although a further explanation was not the scope of the present study with its clinical practical approach, it is striking that the mean absolute TLR4 expression after LPS is even down-regulated compared with un-stimulated controls in the LR group. Conditions with endotoxin hypo-responsiveness are well described[87, 88].

The capacity for LPS responsiveness has been shown to be related to the variation of individual TLR expression levels[89].

In septic patients with primarily enhanced TLR4 expression a ceiling effect without further

capacity for enhancement of TLR expression has been described[90].

Primarily enhanced TLR4 expression could theoretically limit further TLR up-regulation. However, to mimic the long-lasting influence of OK-432 on monocytes in LMs, we incubated the cells with LPS in vitro for 20 h. In most published studies investigating TLR responsiveness, LPS incubation time is much shorter.

To supplement previous results of the predictive value of dynamic up-regulation of TLR4 after OK-432 sclerotherapy, also absolute values appear to be important. Determination of thresholds could simplify the prediction of successful treatment with OK- 432.

Clinical outcome of patients with LM treated with OK-432 (Paper III)

Paper III reports the largest single center experience with OK-432 treatment for LMs. The time to follow up was longer than previously reported. The outcome of OK-432 treatment was good or excellent in a majority of patients with LM. A few patients with malformations involving the mediastinum and abdominal/ retroperitoneal cavities were of special concern. All patients requiring emergency surgery had involvement of the mediastinum and, or, the abdominal/retroperitoneal cavities (Fig 16). If the LMs affect vital organs, with life-threatening symptoms, acute operative debulking may be needed. If total excision is not possible, complementary sclerotherapy may be mandatory. These malformations often require treatment during infancy. This group of patients had major adverse effects of OK-432 treatment with severe swelling.

Fig 16 Magnetic resonance imaging of a retroperitoneal lymphatic malformation.

Surgery used to be the traditional primary treatment modality for LM and it is still mandated under special circumstances[63]. It is often difficult to excise these lesions due to diffuse infiltration in adjacent tissues and thus high risk of surgical complications[63]. Various non-surgical options have been suggested to treat LM, such as radio frequency ablation, laser and sclerotherapy with different sclerosing agents. Sclerotherapy is the primary treatment modality in most centres[49, 51, 63, 64, 91]. Acevedo et al carried out a systematic review of the literature on non-surgical treatment of lymphatic malformations.

The literature strongly suggests that the majority of patients who undergo sclerotherapy with OK-432 or bleomycin as first-line therapy for head and neck LM will achieve a good to excellent clinical response. Serious complications and the need to progress to surgical salvage were infrequent[48].

The patient characteristics of our cohort were similar to those reported from a similar tertiary care center. However, compared to the cohort of Burrows et al, mixed lesions were more common in our series[50].

One explanation could be that our patients were more strictly classified as mixed if the LM consisted of both micro cysts and macro cysts. In our cohort, 59% of LMs were diagnosed before one-year-of-age and 18% after the age of three. Thus LM is a challenge for pediatric healthcare and with better antenatal screening the number of prenatally diagnosed lesions will probably increase.

Our study had limitations due to the heterogeneity of the LM patient characteristics and the fact that it is a retrospective study. Multivariate analysis showed a significant negative impact on the clinical outcome for only three of the tested variables: previous treatment, lesion location and the number of injection treatments. The negative effect on outcome by previous treatment, using other sclerosing agents or surgery, was in concordance with previous studies and may have been caused by scarring in the tissue induced by other sclerosants[51].

The location of the lesion predicted unfavorable outcomes, which may be explained by the association between head and neck lesions and mediastinal involvement. These LMs are prone to airway compromise and compression of major central blood vessels. Furthermore, facial lesions often required repeated OK-432 injections with no positive effect on the clinical outcome. There was a clear negative correlation between the number of treatments and

unfavorable clinical outcome, which might have been expected. More than four injections seemed to predict an unfavorable outcome and indicated the need for surgery. Most of the patients with macrocystic LMs (n =27) had an excellent outcome as a result of OK-432 treatment. However, despite similar radiological findings, four of them required more than three injection treatments to get optimal results. Five of the 13 patients with microcystic LM had a good or excellent outcome. These patients had similar radiological findings but smaller lesions compared to those with less favorable outcomes. A similar pattern was seen in the group with mixed lesions (n= 91). The type of LM, microcystic, macrocystic or mixed lesions, is often considered to be a prognostic factor for the outcome of sclerotherapy[67, 68].

The different outcomes in patients with the same type of lesion may indicate that individual factors have an influence on the response to the treatment. One explanation for the variation in clinical response to OK-432 treatment may be individual immune-pharmacological prerequisites[57, 58].

In Paper I and II we showed that dynamic TLR-4 expression on monocytes might represent a predictive parameter for the response to OK-43 in LMs. The long-term outcome of treatment with OK-432 is comparable to short-term outcome. The strength of this study was the fact that it was a large consecutive cohort of LM patients receiving care at one tertiary care facility by the same pediatric surgeons following the same protocol. MRI is a well-recognized method for evaluating patients with LM after sclerotherapy[50]. However, MRI is invasive in pediatric patients, due to the need for general anesthesia and it is also expensive. We chose to evaluate the patients clinically, using CAS as a follow-up instrument. CAS as an evaluation tool is a cost-effective, non-invasive method of evaluating patients. The clinical findings were also the major factor for deciding what treatment the patient should receive.

Treatment algorithm for LM involving the mediastinum (Paper IV)

Patients with LM involving the mediastinum have a high risk for severe complications following sclerotherapy. The swelling is unpredictable and requires extended observation at an ICU with ventilation support. Surgical excision of LMs in the mediastinum can be done with low incidence of complications[92-94].

In our group of patients 71% (5/7) had airway obstructive symptoms at the time of diagnosis. When the trachea was affected it was evident that the distal part of trachea was the most affected part, hence a tracheostomy wouldn't have been protective if additional swelling would occur.

The management of patients with LM in the mediastinum is complex. A major challenge is the timing of the interventions. In our patients airway obstructive symptoms were the main indication for intervention. Nonsurgical options for the treatment of LMs, including sclerosing agents, aspiration, carbon dioxide laser, and conservative management with observation, are widely used. Nevertheless, the multitude of treatment options indicates that there is lack of consensus regarding the best therapy for this disease. Sclerotherapy has the advantage of being a minimally invasive technique, and it is considered to be the first line of treatment for most LMs[35, 49].

Three of the patients treated with sclerosing agents in this study experienced severe complications with additional swelling compromising vital functions. We tried to tailor the sclerotherapy by using different kinds of sclerosing agents (OK-432, bleomycin, doxycycline, sotradecol) in order to reduce the risk of complications based on the unique characteristics of the substances. Despite this all patients experienced grade IV complications according to the Clavien-Dindo classification and required post sclerotherapy intensive care in median eleven days (range 8–31).

In the operated cases the malformation was removed from the mediastinum and it could be confirmed that the compression from the trachea or the lungs was released. On last MRI at follow-up, 4 of 5 operated patients had no remaining LM in the mediastinum and one patient had clinically insignificant micro-cystic rests remaining. The two patients that only received sclerotherapy both had remaining rests of the malformation in the mediastinum surrounding vital structures. There is a clear advantage for the operated patients as the risk of obstruction of the airways or compression of the central vessels is anatomically removed and hence the need of monitoring and follow-up is reduced in comparison to patients treated with sclerotherapy.

The Clavien-Dindo classification system has been showed to be an excellent tool for systematic evaluation of complications after surgery[82].

The decision of which treatment modality a patient should receive must be made based on the best possible outcome with the least degree of severe complications. Thus it is of highest importance to evaluate different treatment modalities with the same classification system.

We showed that patients treated with sclerotherapy and operated patients had comparable number of mild complications; Clavien-Dindo grade I-II complications. However, severe complications, Clavien-Dindo grade III–IV, were more common after sclerotherapy

(median seven, range 6–12) than after surgery (median two, range 1–4).

Surgery has limitations and must be cautiously planned not to jeopardize vital structures. Several authors suggest surgery as a safe and feasible strategy in the treatment of LM[93, 95, 96].

Thoracoscopic resection of intra-thoracic LMs can be feasible and reasonable for surgeons with advanced laparoscopic skills[95].

Although it is feasible to resect intra-thoracic LM with thoracoscopic approach it remains to be properly evaluated if the minimally invasive technique adds advantages to the surgically treated patients. In our group of patients we noticed a longer ICU time after sclerotherapy (median 11 days, range 8–31) than primary surgery (Median 5 days, range 3–8). The patients with sclerotherapy also required several treatments (median 3, range 1–9). Prolonged PICU care is associated with complications such as septicemia, pneumonia and thrombosis associated to central venous catheters[97].

These complications must also be a consideration when managing this group of patients. Based on our experience in managing these challenging cases, we suggest a treatment algorithm for cervical LMs shown in Fig 17. This algorithm is an attempt to guide in the decision-making when dealing with these rare malformations.

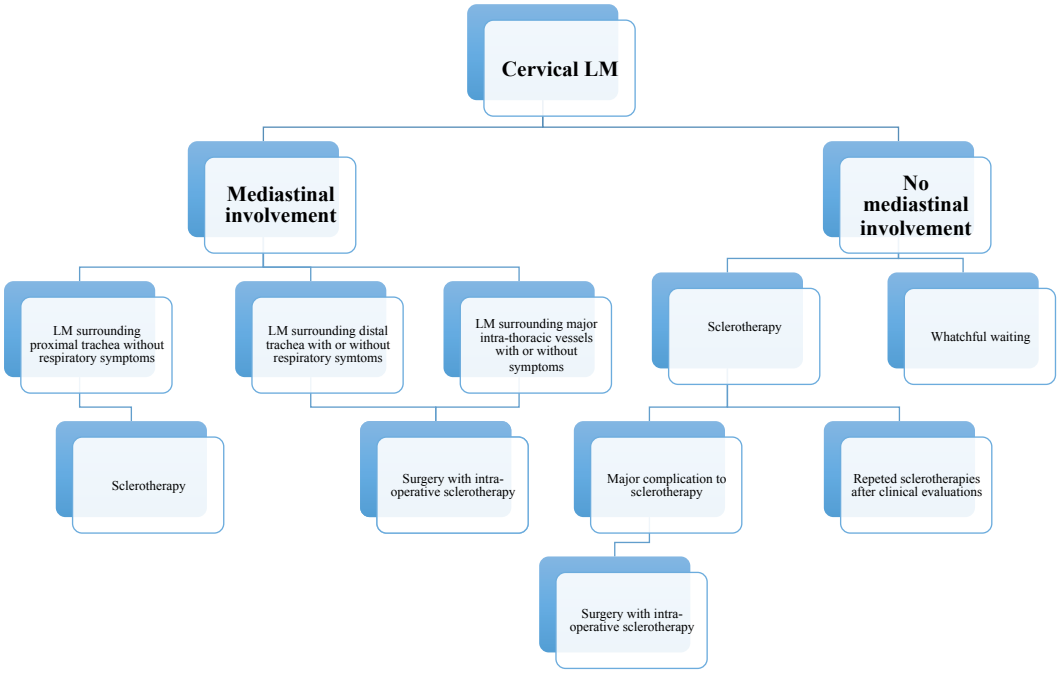


Fig 17 Treatment algorithm for LM involving the mediastinum

LM in the head-neck area that require several treatments are prone to have affected HRQOL (Paper V)

Children and adolescents with LMs report similar HRQOL as the general population of the same age and gender. However, compared to the other patients, those with a head-neck localization of the malformation, and those with a poor treatment result presented lower HRQOL in all dimensions. Also, a high number of treatments was a negative predictor regarding HRQOL. These findings indicate that patients with visible and potentially psychosocialy stigmatizing LMs that require several treatments represent a risk group for an affected HRQOL. Similar studies concerning HRQOL in patients with LM in the head and neck region are rare, and this study of Swedish patients is therefore an important contribution to this field. We found KIDSCREEN-52 to be a useful tool in the extensive evaluation of HRQOL dimensions. HRQOL has previously been studied in children with other serious conditions such as type 1 diabetes mellitus and cancer[98-100]. Childhood cancer survivors reported HRQOL outcomes that mostly parallel those of matched or non-matched healthy comparisons[98, 101]. However, certain diagnostic subgroups have been found to have impaired HRQOL. Diabetes patients, female sex, poor treatment adherence and certain family factors were associated with affected HRQOL[100]. To some extend findings regarding HRQOL among vascular malformation patients diverge. Some studies show that patients with vascular anomalies, or other rare diseases, despite treatment outcome, have an increased risk of physical and psychological undesirable long-term consequences[77-80]. However, in the limited information the literature concerning HRQOL in LM or VM patients, some studies imply that the HRQOL is not affected[102, 103]. Two Finnish studies report HRQOL in VM and LM patients. One of these addressed VM localized to head/neck and included 20 patients evaluated after endovascular sclerotherapy[103].

Assessments included a 20-item quality of life measure. Quality of life outcomes were found to be generally positive, and paralleled to those of aged-matched general population individuals. Quality of life varied with localization of VM, and patients of lower age at treatment start (<16 years), and patients clinically followed by physicians specialized in VM had a better quality of life. Also, the results from the other Finnish study, which surveyed quality of life after head/neck or trunk localized LM treatment, indicated positive quality of life outcomes[81].

In our study, patients reported slightly higher HRQOL compared to the European reference values, while there were no difference in comparison to the Swedish reference data. This particular finding could possibly to some extent be explained by the fact that previous

studies have shown that Swedish population report higher mean values in KIDSCREEN sub-dimensions, physical well-being excluded, than the European reference population[104].

We included all patients with the diagnosis of LM. The median size of LM in the cohort was 40 ml, which means that the size of the malformation in most patients was fairly small. Hence, the influence on the HRQOL could be expected to be limited. However, multiple treatments with sclerotherapy was shown to be associated with lower HRQOL outcomes in a number of sub-dimensions. Although our findings indicate that small malformations can be expected to have limited influence on HRQOL, multiple treatments tend to affect the quality of life negativly. These findings support previous observations that patients requiring multiple treatments report worse quality of life than patients treated with fewer treatment sessions[81].

A clinical implication of the findings of this study is that small malformations prone to several sclerotherapies should be considered for alternative treatment such as surgical excision.

A limitation related to the assessment of HRQOL in this group of patients was the fact that the patients in many cases were treated at the age of 3-8 years, and followed up as late as five years later. The passing of time could be a contributing explanation to the seemingly limited impact on the main HRQOL outcomes. Time might play a role for restoring HRQOL after a possible early negative impact of illness on quality of life among these patients.

4.4 CONCLUSIONS

Paper I and II

The capacity of up-regulation of TLR-4 on monocytes after exposure to the TLR4 ligand LPS represents a predictive parameter for the outcome of treatment of lymphatic malformations with OK-432.

Patients with low response to OK-432 treatment had high TLR4 expression values in monocytes without differences after LPS stimulation. Treatment results can possibly be predicted by absolute values before LPS stimulation.

Paper III

OK-432 sclerotherapy is safe and results in successful outcome in 70% of patients with lymphatic malformations. The long-term outcomes are comparable to short-term outcomes.

Malformations involving the mediastinum and abdominal/retroperitoneal cavities are of special concern with an increased risk of compromise of the airways or the digestive tract and should be considered for primary surgery.

A significant negative impact on the clinical outcome for previous treatment, lesion located to head-neck area and the number of injection treatments. More than four injections seem to predict an unfavorable outcome and indicate the need for surgery.

CAS as an evaluation tool is a cost-effective, non-invasive method of evaluating patients.

Paper IV

Patients with LM involving the mediastinum have a high-risk for severe complications following sclerotherapy.

The complications after surgery as well as sclerotherapy could by analyzed according to Clavien-Dindo classification for postoperative complications. Severe complications, Clavien-Dindo grade III–IV, are more common after sclerotherapy than after surgery

The swelling after sclerotherapy is unpredictable and the treatment requires extended

observation at an ICU with ventilation support.

Tracheostomy does not prevent the risk for tracheal compression in mediastinal LM.

We recommend surgical resection of the LM in the mediastinum, with the possibility of intra-operative sclerotherapy as an adjunctive. Surgical excision of LMs in the mediastinum can be done with low incidence of complications.

Paper V

HRQOL in patients with LMs is similar to standardized population norms.

Patients with LM localized in the head and neck area requiring multiple treatments constitute a risk group for affected HRQOL and was shown to be associated with lower HRQOL outcomes in a number of sub-dimensions. Multiple treatments *per se* imply a risk for HRQOL impairment.

A clinical implication of the findings of this study is that small malformations prone to several sclerotherapies should be considered for alternative treatment such as surgical excision.

5. FUTURE PERSPECTIVES

In modern medicine several specialties narrow and specialist focus on smaller details as the knowledge within the fields expand. A consequence of this evolution is that gaps in knowledge may occur in between the focus areas. A need of a helicopter perspective unifying all knowledge and closing the gaps emerge. The field of vascular anomalies remains to be further explored. The entity involves a variety of rare congenital vascular lesions that may occur in all part of the body and require multi-disciplinary cooperation's. It is essential that cross-national as well as international societies such as ISSVA, NSVA (Nordic Society for the study of Vascular Anomalies) and the VascERN (Vascular European Reference Network) with representatives from all involved specialities continue the work of making consistency regarding the nomenclature in order to facilitate for clinicians and scientists across this wide field to communicate using a common language specific for each entity.

In my future scientific work I will continue with two epidemiological studies.

The first study will be a population-based case-control study consisting of all children born in Sweden during the study period from1/1 1973 to 31/12 2014 according to the Medical Birth Register. The cases will be identified in the National Patient Register and consists of all children with lymphatic malformations in the study cohort. Controls will be randomly selected from the cohort without lymphatic malformations (5 controls per case) matched for sex and year of birth. The following maternal risk factors will be analyzed; maternal age, maternal BMI, mother´s medical history, smoking and medications. The perinatal demographics that will be studied are; the place of birth, twin pregnancy, gestational age, birth weight, birth height, and associated malformations in the baby. The data received will be completely anonymous. The association between exposure and risk will be analyzed. Adjustment for potential confounders will be performed. The incidence of lymphatic malformations per number of live births will be calculated for each calendar year.

The second study will be a register study with data from the Medical Birth Register, Patient Register, the Register for Prescribed Medicine, the Cancer- and Cause of death Register. This will be a cohort study, which consists of all children born with lymphatic malformations from 1/1 1964 to 31/12 2014 according to the Medical Birth Register and the National Patient Register. Data on associated diseases will be collected from the Patient Registry, Cancer

Register, Cause of Death Register and the Register for Prescribed Medicine. Register data are linked using the social security number. The received data will be completely anonymous. Data will be analyzed for various diseases and their prevalence in patients with lymphatic malformations. Medication may be an indication of a disease. Controls will be randomly selected children from the cohort without lymphatic malformations (5 controls per case) matched for sex and year of birth.

Epidemiological register studies do have special ethical reflections. Register studies in Sweden do not mandate written informed consent. From the researchers point of view it is obvious that many register studies wouldn't be possible to perform if the legislation would change since many register studies are so extensive which prohibits the possibility to inform each individual in the study and receive written consents. However, as a scientist one must know that the received data, in spite of being anonymous to the investigators, may be linked to specific individuals and should thus be protected and handled as classified information.

5 APPENDIX

PART 2 (KSCR). 8-18 y
To be completed by the one who has been sick; the child him/herself (with assistance if needed)

Date: → ____ ____ *Month Year*

Hello,

How are you? How do you feel? This is what we would like you to tell us.

Please read every question carefully. Which answer comes to your mind first? Choose the box that fits your answer best and cross it.

Remember: This is not a test so there are no wrong answers. It is important that you answer all the questions and also that we can see your marks clearly. When you think of your answer please try to remember the last week.

You do not have to show your answers to anybody. Also, nobody who knows you will look at your questionnaire once you have finished it.

1. Physical Activities and Health

1. **In general, how would you say your health is?**
   - ☐ excellent
   - ☐ very good
   - ☐ good
   - ☐ fair
   - ☐ poor

Thinking about the last week ...

	not at all	slightly	moderately	very	extremely
2. Have you felt fit and well?	☐	☐	☐	☐	☐
3. Have you been physically active (e.g. running, climbing, biking)?	☐	☐	☐	☐	☐
4. Have you been able to run well?	☐	☐	☐	☐	☐

Thinking about the last week ...

	never	seldom	quite often	very often	always
5. Have you felt full of energy?	☐	☐	☐	☐	☐

2. Feelings

	Thinking about the last week ...	not at all	slightly	moderately	very	extremely
1.	Has your life been enjoyable?	☐	☐	☐	☐	☐
2.	Have you felt pleased that you are alive?	☐	☐	☐	☐	☐
3.	Have you felt satisfied with your life?	☐	☐	☐	☐	☐
	Thinking about the last week ...	**never**	**seldom**	**quite often**	**very often**	**always**
4.	Have you been in a good mood?	☐	☐	☐	☐	☐
5.	Have you felt cheerful?	☐	☐	☐	☐	☐
6.	Have you had fun?	☐	☐	☐	☐	☐

3. General Mood

	Thinking about the last week ...	never	seldom	quite often	very often	always
1.	Have you felt that you do everything badly?	☐	☐	☐	☐	☐
2.	Have you felt sad?	☐	☐	☐	☐	☐
3.	Have you felt so bad that you didn't want to do anything?	☐	☐	☐	☐	☐
4.	Have you felt that everything in your life goes wrong?	☐	☐	☐	☐	☐
5.	Have you felt fed up?	☐	☐	☐	☐	☐
6.	Have you felt lonely?	☐	☐	☐	☐	☐
7.	Have you felt under pressure?	☐	☐	☐	☐	☐

4. About Yourself

	Thinking about the last week ...	never	seldom	quite often	very often	always
1.	Have you been happy with the way you are?	☐	☐	☐	☐	☐
2.	Have you been happy with your clothes?	☐	☐	☐	☐	☐
3.	Have you been worried about the way you look?	☐	☐	☐	☐	☐
4.	Have you felt jealous of the way others look?	☐	☐	☐	☐	☐
5.	Would you like to change something about your body?	☐	☐	☐	☐	☐

5. Free Time

	Thinking about the last week ...	never	seldom	quite often	very often	always
1.	Have you had enough time for yourself?	☐	☐	☐	☐	☐
2.	Have you been able to do the things that you want to do in your free time?	☐	☐	☐	☐	☐
3.	Have you had enough opportunity to be outside?	☐	☐	☐	☐	☐
4.	Have you had enough time to meet friends?	☐	☐	☐	☐	☐
5.	Have you been able to choose what to do in your free time?	☐	☐	☐	☐	☐

A STUDY OF HEALTH AND QUALITY OF LIFE

Children and adults who have been treated for lymphatic malformation
Vs 8-18 years

Ghaffarpour, Claesson, Boman
Karolinska institutet and Astrid Lindgren Children's Hospital
2011

Code number: _____

SURVEY OF LYMPHATIC MALFORMATION (LYMPHANGIOMA)

Ghaffarpour, Claesson, Boman

For children and for adults who received treatment for Lymphatic malformation

At Astrid Lindgren Children's Hospital, in Stockholm we are gathering information of children / adults with Lymphatic malformation. The collected data will provide a description of the disorder and enable us to provide better information about the prognosis. For this aim we need your help.

For many of you, the incident is 10-15 years ago and it may be difficult to remember all the details. We have certain information in our records but we kindly ask you to answer the questions as well as you can.

For the children, of course, their parents or other relatives may answer the questionnaires. Also for those over 18 years old, we recommend that you may ask other family members to get the complete picture about what happened, the treatment. and the results of the treatment.

But it is important that patients' own experiences will appear in the answers to questions regarding received information, experiences of the disease, and possible problems. In the questionnaire is inserted a notion at those parts where children/patients themselves who should respond – although with help from parents for the younger, if needed. For the youngest children under 8 years old, parts of the questionnaire (Part 2) come in specific parent-response versions.

The date when you filled out the questionnaire: ENTER DATE HERE →

PART 1

Tick off the boxes for the options that apply to you at the next questions.
You can type some of the answers directly into the questionnaire form in your computer, those places are marked with yellow colour.

01	Do you remember your first contact with us (ie Gösta Claesson and/or Nader Ghaffarpour)	YES 1☐	NO 2☐		
02	Is there anything you particularly remember about what happened?	YES 1☐	NO 2☐		
03	If you answere "yes" at previous question, enter what you particularly remember → **ENTER HERE →**				
04	How did you get in contact with us? 1☐ By general practitioner (GP) 2☐ Internet 3☐ Other means, please specify here **WRITE HERE →**				
05	How long time did it take from your first symptoms until you were diagnosed?	less than 3 months 1☐	3-6 months 2☐	6-12 months 3☐	more than 1 year 4☐
06	How many doctors did you meet before you came here / got in touch with us?	1-3 1☐	4-6 2☐	7-10 3☐	>10 4☐

07	What was the reason for you to come to us/ get in touch with us (i.e. LM representatives of the pediatr. surg. unit) ? 1☐ Concerns about the lump 2☐ Acute onset 3☐ Pain 4☐ Temporary signs (eg ultrasound diagnostics) 5☐ Breathing problems 6☐ Other reason, please specify: **WRITE HERE →**
08	How was the information about your treatment? Very good 1☐ Good 2☐ Neither good nor bad 3☐ Poor/Bad *) 4☐
09	*) If the information was inadequate or poor: specify how? what was missing?: **SPECIFY AND WRITE HERE →**
10	What did you think of the waiting time for the treatment? 1☐ not too long 2☐ quite a long time 3☐ too long 4☐ unacceptable
11	Did it correspond to your expectations? Yes 1☐ No 2☐
12	Do you remember how many injections you got? (The product is called OK-432). You can estimate the number if you are unsure → **NUMBER →**
13	*If you **just** got injected with OK-432 **at the same time** the "lymphangioma lump" **was operated on,** you need **not** answer this following question.* How great was your reaction after the **first** injection on a scale 0 -10? Tick off the symptoms that apply to you (big box) and then tick off the small box, which stands for the number that corresponds to you (everything in between: 0 = no symptoms, 1 = minimal, 2 = small, and so on to 9 = high, 10 = very great problems) → 1☐ Swelling - Grade → 0 1 2 3 4 5 6 7 8 9 10 4☐ Ache / Pain - Grade → 0 1 2 3 4 5 6 7 8 9 10 2☐ Redness - Grade → 0 1 2 3 4 5 6 7 8 9 10 5☐ Malaise - Grade → 0 1 2 3 4 5 6 7 8 9 10 3☐ Fever - Grade → 0 1 2 3 4 5 6 7 8 9 10 6☐ **Other** symptoms → **Other** symptoms → **WRITE other symptoms here →** - Grade → 0 1 2 3 4 5 6 7 8 9 10

	7☐ **Other** symptoms 2 → SPECIFY HERE→ - Grade → 0 1 2 3 4 5 6 7 8 9 10 ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐	8☐ **Other** symptoms 3 → SPECIFY HERE→ - Grade → 0 1 2 3 4 5 6 7 8 9 10 ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

14	*Question No.14 should **not** be answered by those who only received **one single** injection treatment..* If you had multiple injection treatments, did you have **various** troubles after **various** treatments?				Yes 1☐	No 2☐
15	If you had **varying levels of** discomfort after the treatments: How did you experience your symptoms			They decreased 1☐	They increased 2☐	They shifted 3☐
16	Do you have any **remaining problems?**				Yes 5☐	No 6☐
17	After the treatment, do you have persistent **VISIBLE SIGNS** of your lymphangioma?				Yes 1☐	No 2☐
18	If you still have problems of any kind - how annoying are they for you?	Very little 1☐	A little 2☐	Quite a bit 3☐	Very 4☐	Very much 5☐
19	If you have persistent symptoms, please describe below and indicate for example problems with appearance, intolerance from the environment, education, career choices, etc. **ENTER HERE →**					
20	Overall, how satisfied or dissatisfied are you with your treatment and contact with us? 1☐ Very Satisfied 2☐ Satisfied 3☐ Neither satisfied nor dissatisfied 4☐ Dissatisfied 5☐ Very dissatisfied					
21	Space for general comments and comments about your illness, the information you received, treatment and your life situation **ENTER HERE →**					
22	Please write here what you think is important to improve hospital care and health care concerning lymphangioma. You can make a list of several things **ENTER HERE →**					

23	A☐ I AM 15 YEARS OR OLDER 1 I answered this questionnaire myself 2 In collaboration with adults/ relatives I received assistance in answering, question / questions No → **ENTER HERE** → B☐ FOR PATIENTS BETWEEN 4 AND 14 YEARS 1☐ We (caregivers) answered this questionnaires all by ourselves 2☐ I (care giver) answered this questionnaire all by myself 3☐ in collaboration with our/my child The following questions were answered by our/my child him-/herself **ENTER the number of the questions HERE** → Enter here who helped by answering: i.e, child's parents / mother / father / etc **ENTER HERE** →

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Tomas Wester my principle supervisor. Let me state out the obvious fact that this would not have been possible without your guidance and support in times when I was exhausted beyond reason and did not see how to proceed. I am truly fortunate to have you as my scientific leader, boss, clinical role model and mostly as a friend.

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Friends and family, I am lucky to have so many fabulous people in my life. Thank you all for making my life what it is, peace by peace. Please write your name here:…….....

Diego, Anders, Björn, "Show me your friends and I will tell who you are?" I would show you guys….life-witnesses-buddies!

Ramin, my big brother, how can I acknowledge what you have meant to me in one sentence? One picture comes up in my mind from the early years, sitting on my red little bike chasing you in vain on your blue little bike thinking he is the coolest big brother in the world. Still chasing on my little red bike….

Navid, my nephew in life and my little brother at heart. I´m so happy that you are part of my life and that we are finally the same age!

Mamma and Baba. Thank you for my life! My loving mother thank you for showing me that life has no boundaries and encouraging me to believe in my self. You are a true role model showing that everything is possible. Baba, my dear father the first and most important role model as a man and as a doctor. All my achievements in medicine have been done to make you proud here in your new homeland.

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Nicole and Estelle, my darling daughters, I´m so greatful for life that has rewarded me with the largest gift of all; being your father. Thank you for setting the right perspectives of life for me. Every beat of my heart is because of you two.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Lymfatiska missbildningar (LM) definieras som strukturella fel i lymfkärlen som uppstår på grund av defekt fosterutveckling av lymfkärlen. Förekomsten av dessa missbildningar är svår att ange i exakta siffror. I litteraturen varierar uppgifter om förekomsten mellan 1: 6000 till 1:16.000 levande födda.

Orsaken till LM är multifaktoriell. Genetiska studier visar mutationer i somatiska gener så som PIK3CA-genen vara associerad med förekomsten av LM.

Histologiskt är LM godartade, men beroende på lokalisation, storlek och oväntad svullnad kan de orsaka allvarliga komplikationer som hotar vitala funktioner i kroppen så som exempelvis luftvägarna eller synen. En stor svullnad i ansikte eller på halsen kan också påverka estetiken och på så vis utgöra en psykologisk belastning för patienterna och deras familjer. LM är mycket immunreaktiva och är benägna att återkommande bli infekterade. De kan lätt bli inflammerade och orsaka smärta samt kroniska läckande sår. Vissa anatomiska regioner så som brösthålan (mediastinum) är extra bekymmersamma. En plötslig svullnad i mediastinum som utgörs av ett begränsat hålrum med vitala strukturer så som luftvägarna, de stora kärlen och hjärtat kan lätt leda till livshotande komplikationer.

Det finns inte tillräckligt med vetenskapligt underlag för att skapa en tydlig behandlingsalgoritm för handläggandet av LM. Kirurgi har tidigare ansetts som första behandlingsalternativet. Kirurgi har dock sina begränsningar då dessa missbildningar ofta infiltrerar intilliggande strukturer, såsom kärl och nerver. Det gör att total resektion blir svår och potentiellt farligt. Perkutan skleroterapi har ersatt kirurgi som förstahandsbehandling i de flesta fall. Skleroterapi har dock också sina begränsningar och kan orsaka allvarliga komplikationer så som svullnad som påverkar vitala funktioner och ärrbildning.

I denna avhandling sökte vi svaret på ett antal essentiella frågor om patienter med LM. Vi ville ta reda på hur det har gått för patienterna över en långtids uppföljning både avseende kliniskt behandlingsresultat och komplikationer såväl som påverkan på livskvalitet. Vi ville utvärdera injektionsbehandlingar och kirurgi som behandling både individuellt men också inbördes i avsikt att skatta behandlingsresultat och komplikationer från de olika behandlingsmetoderna bedömda med samma utvärderingsinstrument.

Målsättningen med **delarbete I** och **delarbete II** var att studera den immunologiska reaktionen efter behandlingen av lymfatiska missbildningar med OK-432. Med ökad

förståelse för den ville vi identifiera en mätbar parameter för att kunna prognostisera utfallet av behandlingen och på så vis kunna etablera en riktlinje för val av behandlingsmetod. Vår hypotes var att OK-432 aktiverar immunförsvaret genom att vara en ickeselektiv agonist till Toll-Like receptor-4 (TLR-4). Vid en aktivering av TLR-4 sker en uppgradering dels av TLR-4 men också TLR-7. Vid en uppgradering av TLR-7 sker en mer potent immunrespons.

Vi analyserade 11 barn som behandlats med OK-432 för lymfatisk missbildning. Sex barn med icke tillfredställande behandlingsresultat (LR) och fem med bra behandlingsresultat (HR). De två grupperna hade jämförbara missbildningar utifrån radiologisk diagnostik. De två grupperna jämfördes avseende monocyternas förmåga till uppreglering av TLR-4 efter 20h stimulering med Lipopolysaccharid (LPS). LPS är en vedertagen selektiv TLR-4 agonist och aktiverar immunförsvaret i det avseendet på samma vis som OK-432. Vi mätte därefter monocyternas uppreglering av TLR-4 och TLR-7 med Flourescence Activated Cell Sorting (FACS). $P<0.05$ ansågs som statistiskt säkert.

I **delarbete I** fann vi att medeluppregleringen av TLR4 efter LPS stimulering var statistiskt högre i gruppen som haft bra behandlingsresultat (HR) P=0.037).

I **delarbete II** fördjupade vi analyserna från arbetet i delarbete I. Vi analyserade om absoluta värden på TLR4 förekomst på monocyter skulle kunna förutsäga patienternas behandlings resultat efter behandling med OK-432.

Vi studerade absoluta värden av TLR-4 på monocyter innan och efter LPS stimulering. De totala TLR-4 värdena efter LPS stimulering var jämförbart i båda grupperna (HR 1142 ± 652 enheter, LR 839 ± 427 enheter, P = 0.85). Pre-stimuleringsvärden i LR gruppen i jämförelse med HR gruppen var 950 ± 718 vs. 477 ± 341. Vi noterade betydande skillnader av förändringen i medel expression av TLR4 efter LPS (HR 665±683vs.LR-111±605,P=0.08).

Vår tidigare observation i delarbete 1 om en nedsatt uppreglering av TLR4 på monocyter efter LPS stimulering i LR gruppen i jämförelse med HR gruppen kan förklaras med TLR prekonditionering. Detta innebär att patienter med LR har en förhöjd tidigare uppreglering av TLR-4 på monocyterna och på så vis blir uppregleringen mindre efter ytterligare stimulering. Denna observation innebär att absoluta värden med definerade tröskelvärden av TLR-4 skulle kunna prognostisera behandlingsutfall.

I **delarbete III** gjorde vi en systematisk genomgång i en retrospektiv journalstudie av alla patienter som behandlats med OK-432 på ALB under perioden 1998 till 2013. Resultatet av

behandlingen utvärderades kliniskt samt genom en enkät. Vi ville klargöra den generella demografin av patienter med LM i denna cohort samt utvärdera lång-tidsresultatet.

131 av 138 patienter mötte inklusionskriterierna. Den lymfatiska missbildningen var lokaliserad till huvud och hals regionen (60%), bålen (20%) och till extremiteterna (6%). Missbildningen förekom på flera lokalisationer i 14% av fallen. Majoriteten (69%) av patienterna hade mixed LM, 10% hade microcystisk LM och 21% hade macrocystisk LM. I median fick de olika missbildningstyperna 3, 2 och2 behandlingar respektive. Median åldern för första behandlingen var 3,4 år. Bra eller utmärkt behandlingsresultat uppnåddes av 70% av patienterna. Antalet behandlingar, tidigare behandlingar och anatomisk lokalisation i ansikte var negativa prognostiska indikatorer. Långtidsresultatet var jämförbart med korttidsresultatet.

Målsättningen med **delarbete IV** var att utvärdera vår erfarenhet av patienter med lymfatisk missbildning som engagerar mediastinum och föreslå en behandlingsalgoritm för stöd i handläggandet av denna ovanliga missbildning.

Detta var en deskriptiv journalgranskning av alla patienter med lymfatisk missbildning med engagemang av mediastinum som behandlats på ALB mellan 2009 och 2015. Vi analyserade data från patienterna avseende demografi, utredning- och behandlings uppgifter, komplikationer till behandling och behandlingsutfall vid uppföljning. Komplikationer definierades och bedömdes enligt Clavien-Dindo klassifikationen. Det kliniska behandlingresultatet bedömdes på en 4 gradig skala (Excellent-Good, Fair-Poor)

Kohorten bestod av sju patienter. Alla patienter fick injektionsbehandling. Fem patienter opererades, två opererades primärt och tre opererades efter allvarlig komplikation efter injektionsbehandling. Patienter som behandlats med injektions behandling och de opererade patienterna hade jämförbart med milda komplikationer, Clavien-Dindo grad I-II komplikationer. Allvarliga komplikationer, Clavien-Dindo grad III-IV, observerades fem gånger mer efter injektionsbehandling jämfört med kirurgi. Det kliniska behandlingsresultatet var genomgående "excellent" för de opererade patienterna och "Fair-Good" för de som endast fått injektionsbehandling.

Målsättningen med **delarbete V** var att beskriva HRQOL (Health Related Quality Of Life) hos en lång-tidsuppföljd kohort av patienter med lymfatisk missbildning.

Alla patienter som behandlats med sklerosering för lymfatisk missbildning på Karolinska universitetssjukhuset under perioden 1998-2013 ingick i en retrospektiv journalgenomgång.

En för denna studie specifik enkät skickades ut till alla patienter som hade en uppföljning på minst fem år med frågor om kvarvarande symtom och nöjdhet med behandling och omhändertagandet. KIDSCREEN-52 användes för evaluering av HRQOL. KIDSCREEN-52 är ett validerat instrument för att utvärdera samtliga dimentioner av HRQOL.

Fyrtionio av 59 (83%) patienter som mötte studiens inklusionskriterier svarade på enkäten och togs med i studien. Det var statistisk relevant negativ korrelation mellan totalt antal injektionsbehandlingar och Autonomy (p=0.013), Parent Relation and Home Life (p=0.014), Financial Resources (p=0.025), och School Environment (p=0.037).

Lokalisation av missbildning i huvud och hals regionen utgjorde genomgående en negativ prediktor på samtliga HRQOL parametrar med den starkaste korrelationen med Psychological Well-being (p=0.009), Parent Relation and Home Life (p=0.017) och School Environment (p=0.006). Otillfredställande resultat av behandlingen noterades också som en genomgående negativ prediktor på samtliga HRQOL parametrar.

Patienter med synliga tecken på missbildning uppvisade en icke statistiskt säkerställd tendens att vara mindre nöjda med behandlingen (p=0.155). Graden av upplevda besvär av kvarvarande missbildning inklusive synlig missbildning var negativt korrelerat med den övergripande nöjdheten med behandlingen (r=0.384, p=0.011).

De huvudsakliga slutsatserna i de fem delarbetena är:

1. Monocyters förmåga att uppreglera TLR-4 efter LPS stimulering är en positiv prognostisk och mätbar parameter för ett tillfredställande behandlingsresultat av LM med OK-432.
2. Monocyter kan på grund av tidigare stimulering av TL4-agonister ha en uppgradering av TLR-4 och på så vis mattas immun responsen av. Höga pre-stimuleringsvärden av TLR-4 förekomst på monocyter utgör således en mätbar och negativ prognostisk faktor.
3. Antalet behandlingar med sclerosering, tidigare behandlingar och anatomisk lokalisation i huvud och hals regionen var negativa prognostiska indikatorer. Långtids resultatet var jämförbart med korttids resultatet. Fyra icke tillfredsställande scleroseringar bör utgöra gräns för beslut till alternativ behandling så som exempelvis kirurgi.
4. Patienter med lymfatisk missbildning som engagerar mediastinum utgör en risk grupp för att få allvarlig komplikation efter sclerosering. Svullnaden är oförutsägningsbar och kräver lång observationstid på intensivvårdsavdelning med mekanisk ventilation. Tracheostomi kan inte skydda luftvägarna vid svullnad i mediastinum. Kirurgisk excision

av lymfatisk missbildning i mediastinum rekommenderas med möjlighet till intraoperativ adjuvant injektionsbehandling.

5. LM patienter har jämförbar HRQOL som befolknings normdata. Patienter med missbildning i huvud- och halsregionen som behöver multipla behandlingar utgör en riskgrupp för att få negativ påverkan på HRQOL parametrar. Multipla behandlingar i sig utgör en risk för påverkad HRQOL.

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8 PAPER I-V